EVADING GLYCOPEPTIDE ANTIBIOTIC RESISTANCE

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By

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Abstract

Glycopeptide Antibiotics (GPAs) such as vancomycin are often used clinically as antibiotics of last resort against infections due to Gram-positive bacteria that are resistant to more commonly used antibiotics such as methicillin. The clinical emergence of vancomycin resistant enterococci (VRE) and vancomycin resistant *S. aureus* (VRSA) necessitates methods to evade this resistance.

GPAs consist of a heptapeptide backbone that is cross-linked to create a pocket that binds the D-Alanyl-D-Alanine terminus of peptidoglycan intermediates, inhibiting strengthening of the cell wall and resulting in susceptibility to osmotic stress. Resistance to GPAs occurs when D-Ala-D-Lactate replaces D-Ala-D-Ala and the GPA pocket can no longer bind effectively. In order to create novel binding pockets, we must understand the specificity of the P450 monooxygenase enzymes that have been shown to catalyze the cross-links. The 4 P450-encoding genes of the GPA A47934 biosynthetic cluster of *Streptomyces toyocaensis* as well as genes encoding electron transport proteins necessary for P450 function from *Streptomyces coelicolor* were cloned in *Escherichia coli* for heterologous expression and characterization. One P450, StaJ was purified and shown to bind CO as expected using spectrophotometric tests.

The genes responsible for GPA resistance are regulated by a two component regulatory system consisting of a sensor kinase (VanS) and a response regulator (VanR). In order to probe the events leading to VanS autophosphorylation and ultimately resistance activation we utilize a series of GPA derivatives harbouring the photolabile group benzophenone as well as the fluorescent and affinity moieties BODIPY and biotin. Benzophenone permits light controlled covalent binding of the GPA to proteins that bind

them while BODIPY allows fluorescence detection and biotin allows enrichment and detection by Western analysis. We report that this system was insufficient to clearly identify vancomycin binding proteins due to background signals despite multiple rounds of troubleshooting. It must be our conclusion that under the conditions tested, there are no proteins that bind the GPA derivative used in this study.

Acknowledgements

I first would like to thank my mother, without whom I would be lost. Thank you for keeping me focused and moving forward despite many obstacles in recent years. Thank you for your strength and support through all aspects of my life.

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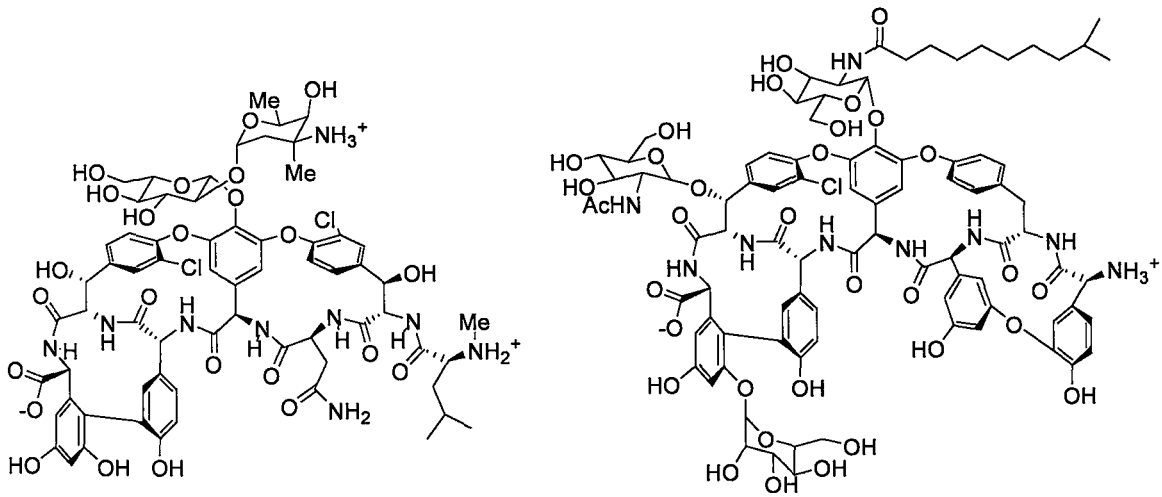
Chapter 1 – Introduction

1.0- Introduction to Glycopeptide Antibiotics

Glycopeptide antibiotics (GPAs) such as vancomycin and teicoplanin have come to be used as antibiotics of last resort for treatment of infections due to multiple drug resistant Gram-positive bacteria such as methicillin resistant *Staphylococcus aureus* (MRSA) (46, 56). Vancomycin was first discovered in the 1950's as a natural product produced by the bacterium *Amycolatopsis orientalis* during fermentation. Teicoplanin was discovered in the early 1980's as part of a mixture of compounds, initially termed teichomycins produced by *Streptomyces teicomyceticus*. The structures of vancomycin and teicoplanin are shown in figure 1.1. Both consist of a heptapeptide backbone containing both proteinogenic and non-proteinogenic amino acid residues. The aromatic amino acids that comprise the backbones are cross-linked to each other to give the molecules structural rigidity and are further modified by glycosylation, methylation, chlorination, and lipidation.



Vancomycin **Teicoplanin**

Figure 1.1- The structures of vancomycin and teicoplanin both consist of heptapeptide backbones that differ in their amino acid composition and further cross-linked and modified by glycosylation, methylation, chlorination and lipidation. Vancomycin has three intra-strand cross-links while teicoplanin has four (4, 57).

GPAs were initially used infrequently in the clinic due to impurities that led to side effects and the need for intravenous administration, but their use grew during the 1980's in response to increased incidence of MRSA (33). In 1986 vancomycin resistance was discovered in enterococcal bacteria (vancomycin resistant enterococci, VRE) and it was feared that the resistance phenotype would eventually be passed to more virulent species such as *S. aureus* (38). This fear was realized in 2002 with the clinical emergence of vancomycin resistant *S. aureus* (VRSA) (32, 46, 70). The increasing incidence of these virtually untreatable infections emphasizes the need for novel antibiotics that retain antimicrobial activity against VRE and VRSA. It is estimated that up to 30% of all enterococcal infections are due to VRE (32). Research on the biosynthesis, resistance and the regulation of resistance has been ongoing for decades, yet many aspects of each remain unclear. The purpose of this study is to further our

understanding of GPA biosynthesis and resistance in hopes of manipulating biosynthetic machinery to generate novel GPAs and to ultimately evade GPA resistance.

1.1-Current knowledge of GPA biosynthesis

The biosynthetic gene clusters responsible for the production of several GPAs and GPA-like molecules have been sequenced and the functions of the gene products elucidated. The main functions required for GPA assembly are: non-proteinogenic amino acid production, assembly of the heptapeptide, intra-strand cross-linking of aromatic amino acid residues, and modification by halogenation, glycosylation, methylation, lipidation or sulfation. Genes responsible for each function have been found in each of the clusters sequenced. The GPA studied in this work is A47934, a teicoplanin-like GPA produced by the soil-dwelling bacterium *Streptomyces toyocaensis* NRRL15001 during fermentation (12, 80). The biosynthetic cluster has been sequenced in our lab and is compared to the sequenced clusters of other GPAs in Table 1 (57).

Table 1-Summary of proteins encoded by the A47934 biosynthetic cluster and comparison to other sequenced biosynthetic clusters

orf	A47934	Cl-E[a]	Bal[b]	com[c]	Dbv[d]	tcp[e]	Proposed Function
1	VanXst	-	-	-	-	Tcp4	D-Ala-D-Ala dipeptidase
2	VanAst	-	-	-	-	Tcp3	D-Ala-D-lactate ligase
3	VanHst	-	-	-	-	Tcp2	Lactate dehydrogenase
4	MurXst	-	-	-	-	Tcp1	D-Ala-D-Ala adding enzyme
5	StaO	-	-	-	-	-	FemABX homologue
6	StaP	-	-	-	-	-	Putative membrane protein
7	StaQ	CZA382.26	-	ComG	Dbv4	Tcp28	Transcriptional regulator
8	Hmo	Hmo	ORF6	Hmo	Dvb1	Tcp38	p-Hydroxymandelate oxidase
9	HmaS	HmaS	ORF5	HmaS	Dbv2	Tcp37	p-Hydroxymandelate synthetase
10	Pdh	Pdh	-	Pdh	Dbv5	-	Prephenate dehydrogenase
11	HpgT	HpgT	PgaT	HpgT	Dbv37	Tcp36	HPG and DHPG aminotransferase
12	DpgA	DpgA	DpgA	-	Dbv31	Tcp30	3,5-Dihydroxyphenylacetyl-CoA synthase
13	DpgB	DpgB	DpgB	-	Dbv32	Tcp31	Enhances DpgA activity
14	DpgC	DpgC	DpgC	-	Dbv33	Tcp32	3,5-Dihydroxyphenylacetyl-CoA oxygenase
15	DpgD	DpgD	DpgD	-	Dbv34	Tcp33	Enhances DpgA activity
16	StaR	-	-	-	-	-	Putative flavoprotein
17	StaS	-	-	-	-	-	Putative DNA binding protein
18	VanSst	-	-	-	Dbv22	Tcp6	Transmembrane histidine kinase
19	VanRst	-	-	-	Dbv6	Tcp7	Two-domain response regulator
20	StaU	CepM	-	ComL	Dbv24	Tcp16	ABC transporter
21	StaA	CepA	BpsA	ComA	Dbv25	Tcp9	NRPS* (modules 1–2)
22	StaB	CepA	BpsA	ComB	Dbv26	Tcp10	NRPS (module 3)
23	StaC	CepB	BpsB	ComC	Dbv17	Tcp11	NRPS (modules 4–6)
24	StaD	CepC	BpsC	ComD	Dbv16	Tcp12	NRPS (module 7)
25	StaE	CepD	ORF1	ComE			Hypothetical protein
26	StaF	CepE	OxyA	ComI	Dbv14	Tcp18	P450-related oxidase
27	StaG	-	-	-	Dbv13	Tcp19	P450-related oxidase
28	StaH	CepF	OxyB	ComJ	Dbv12	Tcp20	P450-related oxidase
29	StaI	CepH	BhaA	-	Dbv10	Tcp21	Nonheme halogenase
30	StaJ	CepG	OxyC	-	Dbv11	Tcp22	P450-related oxidase
31	StaK	-	-	ComH	-	-	Nonheme halogenase
32	StaL	-	-	-	-	-	Sulfotransferase
33	StaM	-	-	-	Dbv28	Tcp25	Putative nonheme iron dioxygenase
34	StaN	CZA382.28	ORF7	ComF	-	Tcp34	Integral membrane ion transporter

[a]Cl-E: chloroeremomycin cluster (71);[b] bal: balhimycin cluster (54); [c]com: complestatin cluster (21), [d]dbv: A40926 cluster (66); [e]tcp: teicoplanin cluster (65); A47934 (57); *NRPS: Non Ribosomal Peptide Synthase.

The structures of the antibiotics referred to in table 1 are summarized in figure 1.2. Note that the overall structures are similar and the modifications to the backbones are the primary difference.

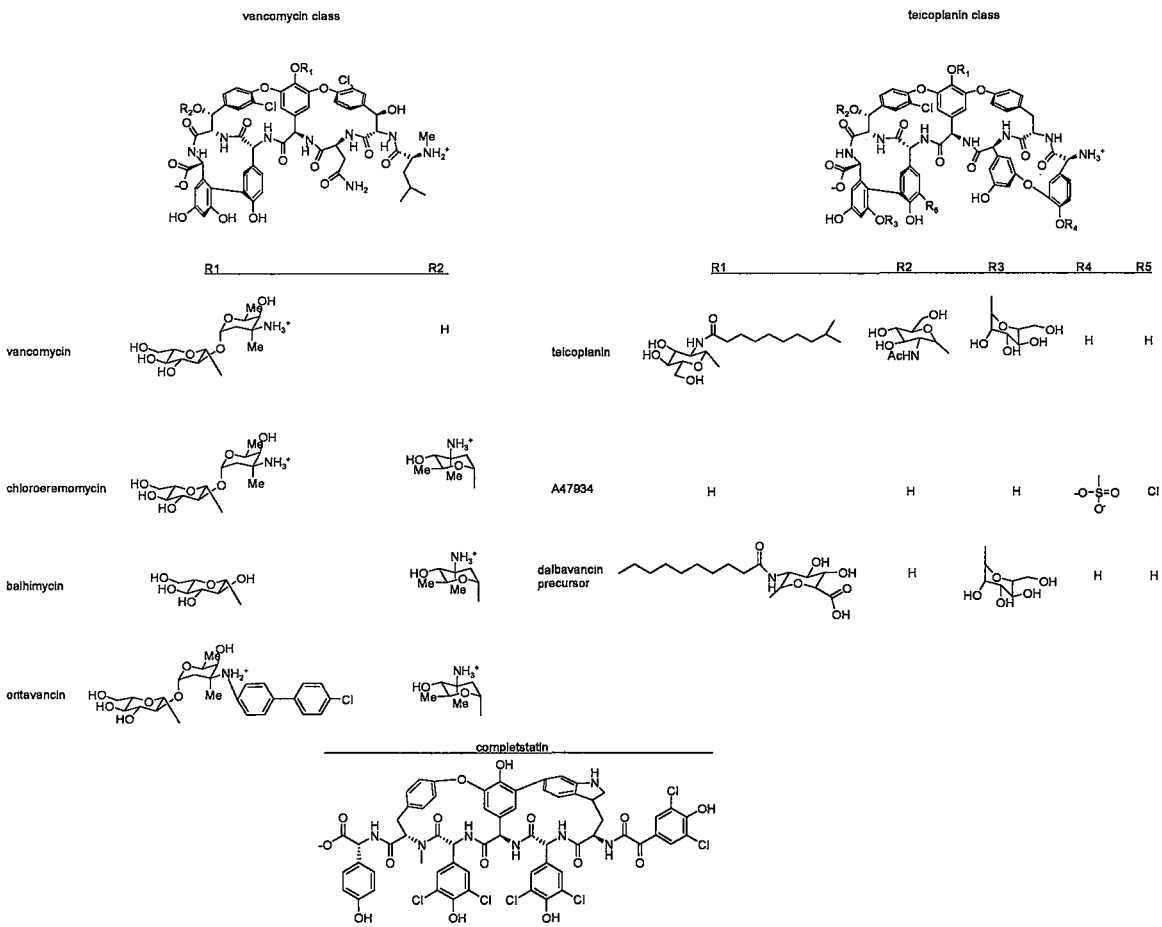


Figure 1.2- Summary of the structures of GPA and GPA-like products referred to in table 1; note how backbones of each class of GPA are similar and thus why many genes are found in all the sequenced biosynthetic clusters to date (5, 55).

A predicted retrobiosynthetic scheme for A47934 is shown in figure 1.3, illustrating each required step for GPA assembly. It is proposed that the unusual amino acids are first produced followed by heptapeptide assembly. The intra-strand cross-links are then made to give the backbone a rigid structure and this aglycone product is the substrate for the modification enzymes, the halogenases and sulfotransferase.

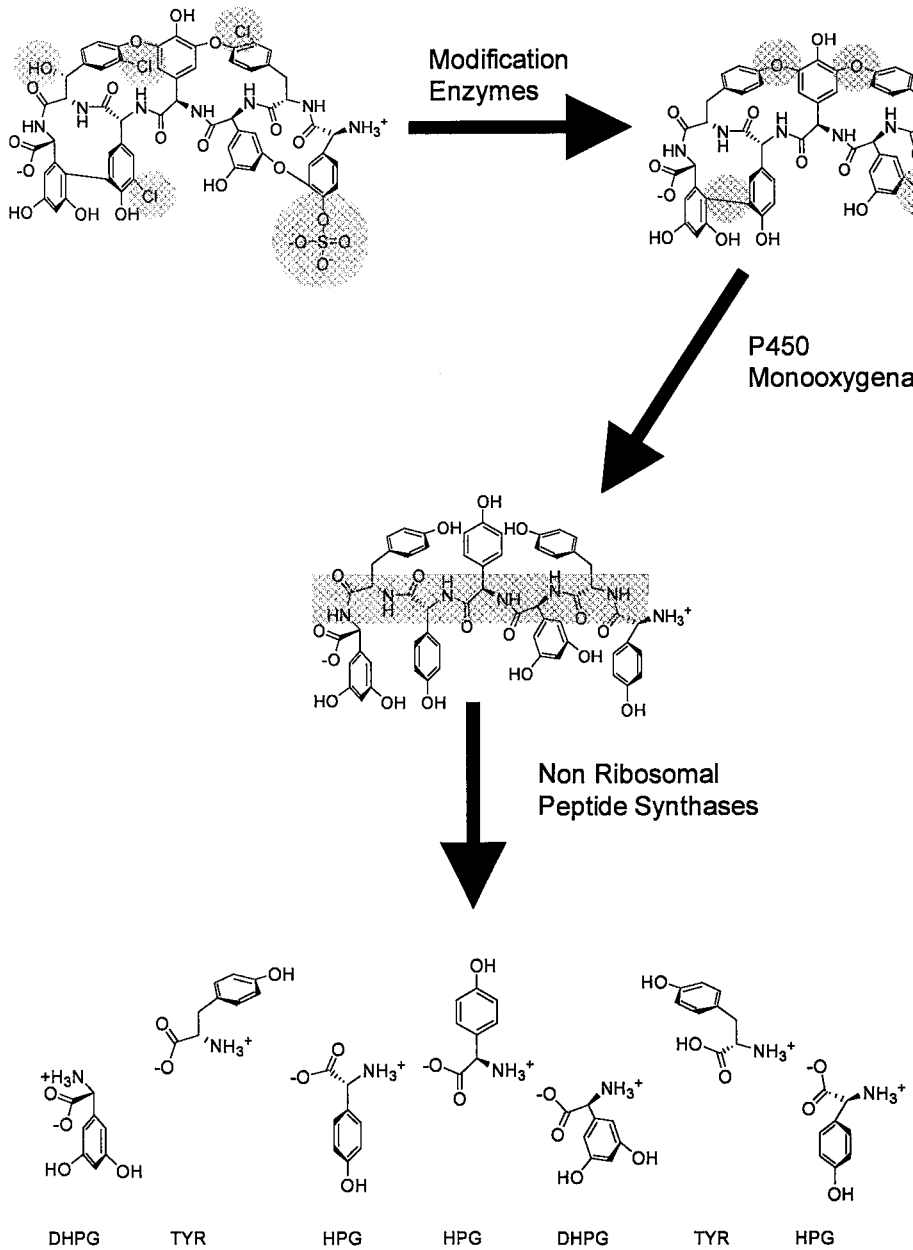


Figure 1.3- Predicted A47934 retrobiosynthesis. HPG: *p*-hydroxyphenylglycine, TYR: tyrosine, DHPG: 3, 5-dihydroxyphenylglycine. It is predicted that the NRPS enzymes first assemble the amino acids into the heptapeptide, followed by cytochrome P450 monooxygenase (P450)-mediated cross-linking of the heptapeptide and finally modification of the aglycone backbone (55).

Each step in GPA biosynthesis will be discussed in more detail in the following sections.

1.1.1-Unusual Amino Acid Biosynthesis in GPA Production

As shown in figure 1.1, GPAs contain a cross-linked heptapeptide backbone that includes both proteinogenic and non-proteinogenic amino acids. The unusual amino acids incorporated in the backbone are 3,5-dihydroxyphenylglycine (DHPG), β-hydroxytyrosine (OH-Tyr) and *p*-hydroxyphenylglycine (HPG). The unusual amino acids HPG, DHPG and OH-Tyr are not produced for normal protein synthesis and are made especially for GPA production by genes encoded within the biosynthetic cluster.

The production of HPG is carried out by a set of four genes found (based on homology) in each of the six sequenced GPA or GPA-like biosynthetic clusters (21, 54, 57, 65, 66, 71). In *A. orientalis* NRRL 18098 (chloroeremomycin cluster), and *Streptomyces lavendulae* (complestatin cluster), the enzyme prephenate dehydrogenase (Pdh) converts prephenate to *p*-hydroxyphenylpyruvate. Hydroxymandelate synthase (HmaS) produces L-*p*-hydroxymandelate from *p*-hydroxyphenylpyruvate that is oxidized by hydroxymandelate oxidase (Hmo) to form *p*-hydroxybenzoylformate. In the final reaction, the aminotransferase HpgT (PgaT in the balhimycin cluster) uses L-tyrosine as an amino donor to make the final products L-HPG and *p*-hydroxyphenylpyruvate, the latter of which can re-enter the cycle, making Pdh necessary only to prime the cyclic mechanism (30). Homologous genes have been found in the A47934 biosynthetic cluster and the nomenclature kept consistent. The predicted reactions are summarized in figure 1.4.

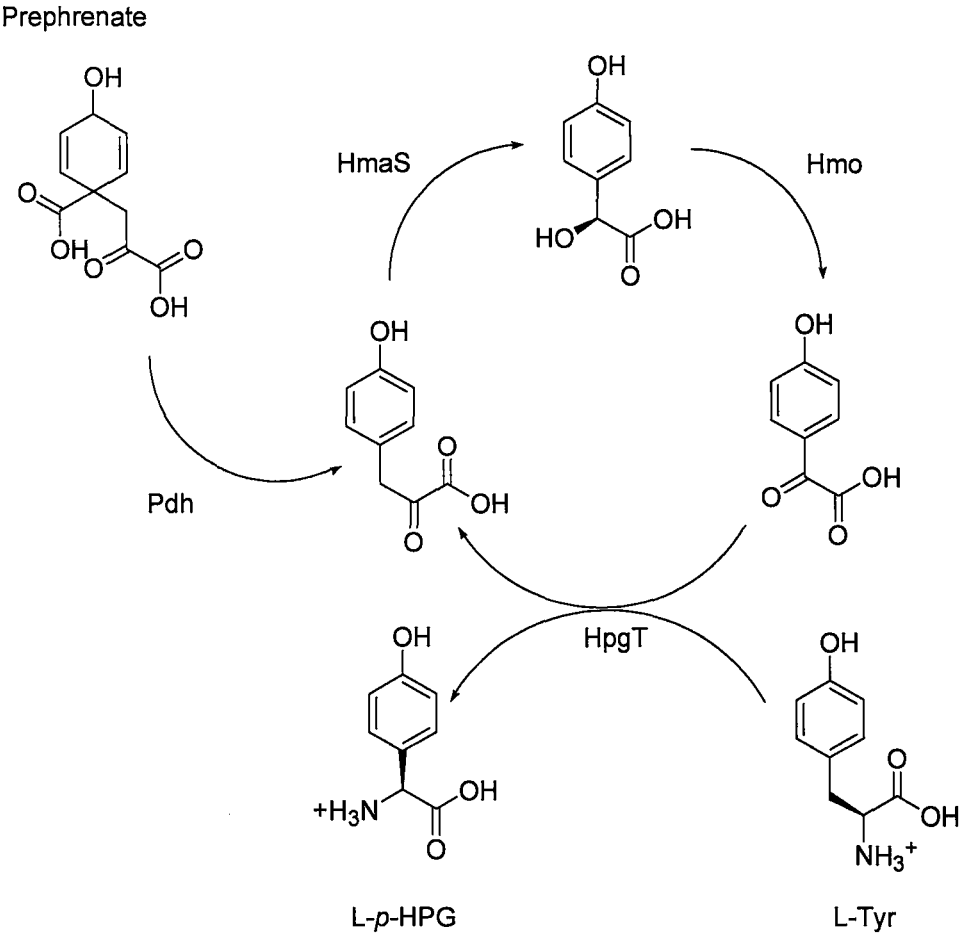


Figure 1.4- *p*-hydroxyphenylglycine biosynthetic scheme illustrating how Pdh primes a three enzyme cyclic mechanism with *p*-hydroxyphenylpyruvate that ultimately yields HPG and another *p*-hydroxyphenylpyruvate molecule. Homologous enzymes are also found in the A47934 biosynthetic cluster. Modified from reference (55).

DHPG synthesis genes are found in all GPA clusters except complestatin (which does not contain DHPG). Work on the *A. orientalis* genes has shown that DpgA links four malonyl CoA molecules giving three CoASH molecules that cyclize to produce 3,5-dihydroxyphenylacetyl-CoA (DPA-CoA). Addition of DpgB and DpgD increases DPA-CoA formation 17-fold and an additional 2-fold respectively. DpgC converts the DPA-CoA into 3,5-dihydroxyphenylglyoxylate, on which HpgT can act (transferring an amino group as in HPG synthesis) to form the final product DHPG (18). The nomenclature was

kept for the homologous proteins in the A47934 biosynthetic cluster. The reactions are outlined in figure 1.5.

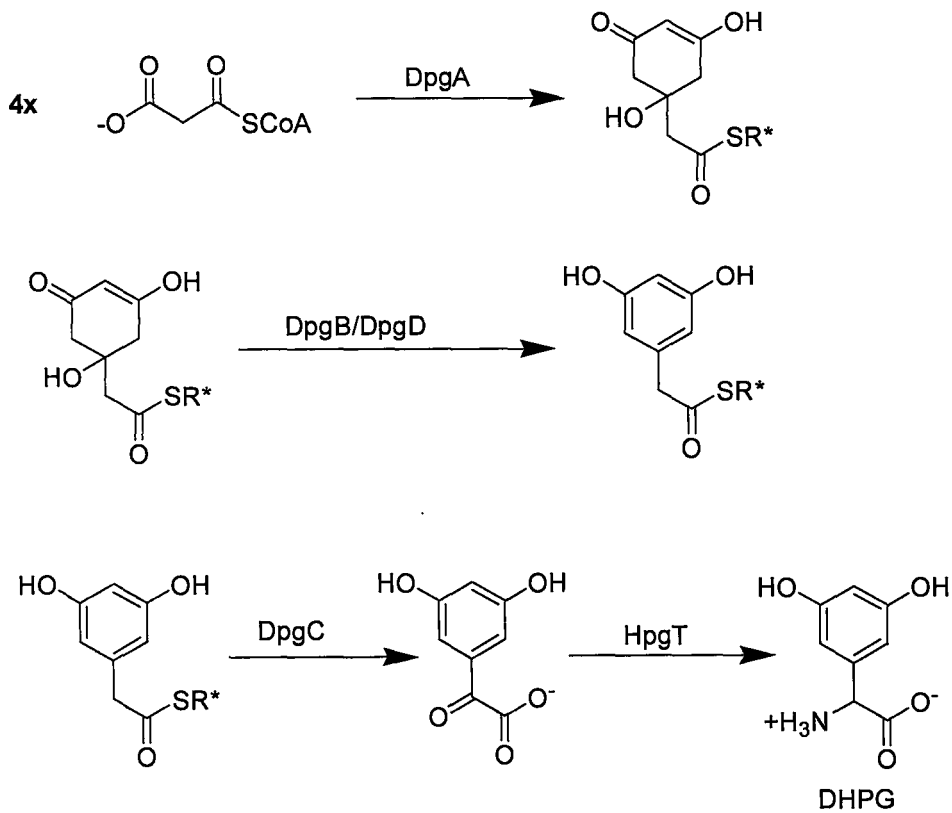


Figure 1.5- 3,5-dihydroxyphenylglycine biosynthesis showing how four malonyl CoA molecules are cyclized and modified to yield DHPG. Note that HpgT is the same enzyme involved in amino group transfer in HPG biosynthesis. Homologous enzymes are found in the A47934 biosynthetic cluster of *S. toyocaensis*. *R=CoA or DpgA. Modified from reference (55).

A NRPS-like enzyme, a P450 hydroxylase and a thioesterase produce OH-Tyr in the chloroeremomycin producer (NRPS domains are explained in following section). The NRPS-like protein adenylates tyrosine and attaches the activated product to a phosphopantetheine linker extending from the thiolation domain where the P450 hydroxylase adds the hydroxyl group which is followed by release by hydrolysis of the thioester bond by a thioesterase (19).

Unlike in other GPA biosynthetic clusters, these three genes were not found in the A47934 cluster and thus OH-Tyr production in *S. toyocaensis* will be the focus of future research. The A domain of the sixth NRPS module in StaC of the A47934 biosynthetic cluster (OH-Tyr) matches the recognition pockets for a Tyr-specific A domain better than a OH-Tyr-specific A domains, leading to the prediction that OH-Tyr may be hydroxylated post-assembly in A47934 production (55). OH-Tyr biosynthesis for novobiocin production is summarized in figure 1.6.

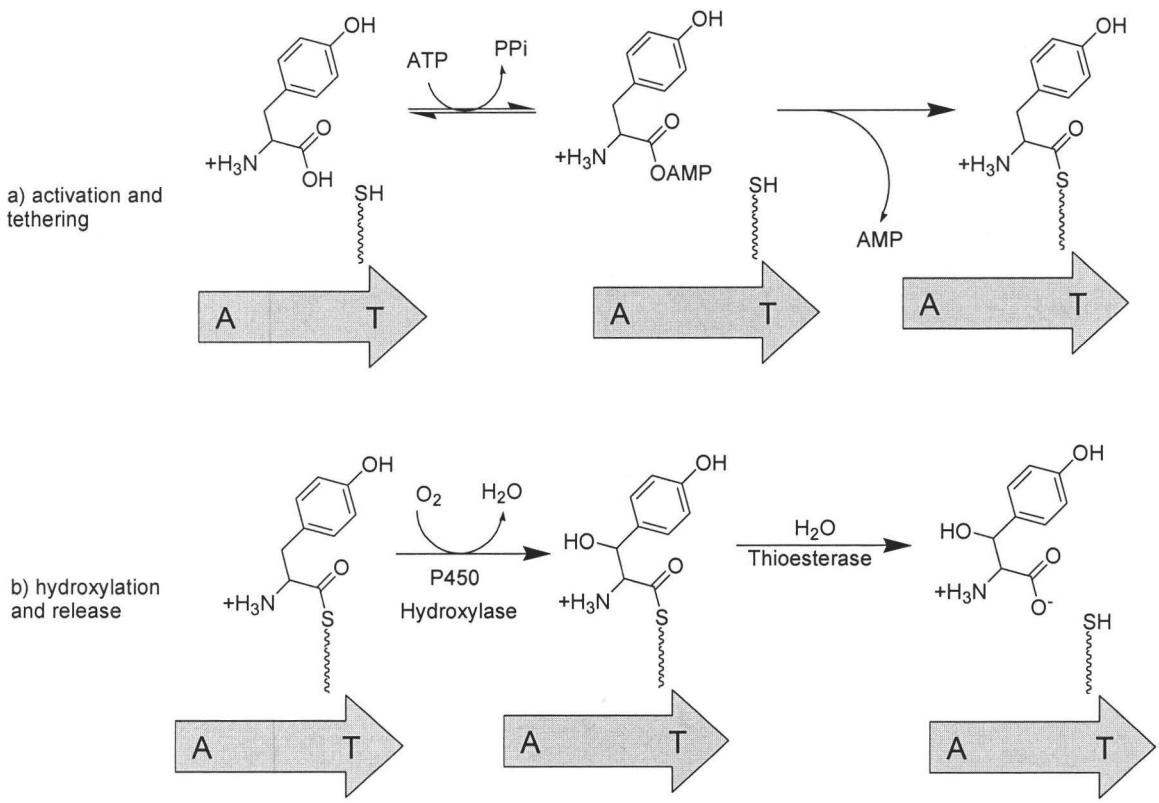


Figure1.6- β-hydroxytyrosine biosynthetic scheme highlighting the NRPS-like protein and P450 monooxygenase responsible for production of β-hydroxytyrosine from tyrosine in the production of novobiocin. Homologous proteins have not been found in the A47934 producer, therefore the source of OH-Tyr in this organism is unknown. Summarized from reference (55).

1.1.2 - Glycopepide Backbone assembly

Because the GPA peptide backbones contain non-proteinogenic amino acids, they are not mRNA encoded and assembled on ribosomes. The vancomycin class GPAs have the backbone sequence NH_2-Leu-OHTyr-Asn-HPG-HPG-OHTyr-DHPG-COOH while the teicoplanin class is composed of NH_2-HPG-Tyr-DHPG-HPG-HPG-OHTyr-DHPG-COOH. The two differ only in the three N-terminal residues. The heptapeptide backbones of GPAs are assembled by large, multi-domain proteins known as non-ribosomal peptide synthases (NRPS). Non-ribosomal peptide synthases are multi domain proteins that assemble the heptapeptide backbone of GPAs and many other biologically active compounds (74).

The three domains mainly responsible for the assembly are the adenylation domain (A), thiolation domain (T) and condensation domain (C). Also present in NRPSs are epimerization domains (E), thioesterase domains (Te) and methylation domains (Me) (74). These domains are repeated once for each residue added, except in the first module (each module contains the domains to add one residue), which does not require a C domain as the succeeding C domain carries out condensation. The domains and their functions are shown in figure 1.7.

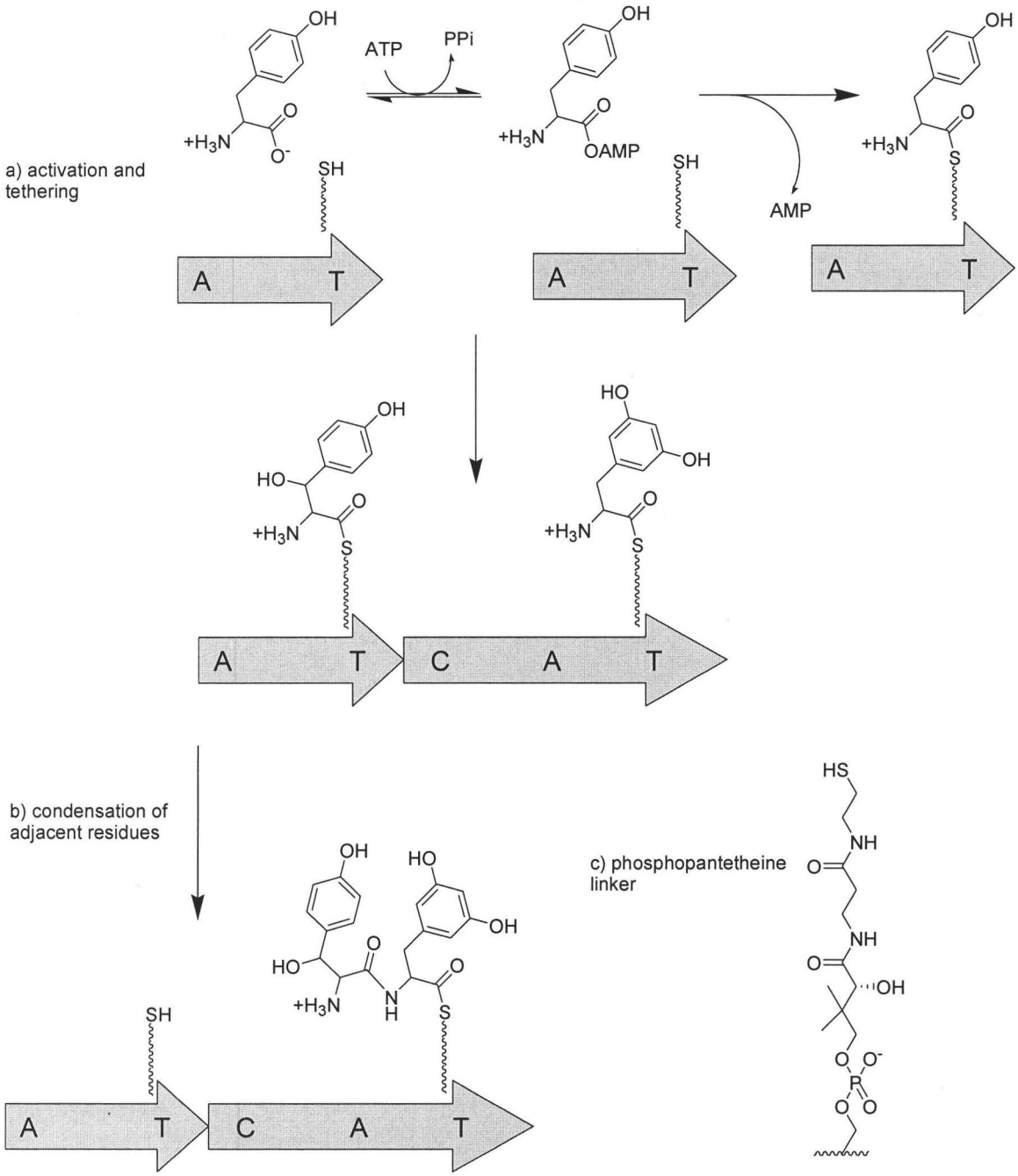


Figure 1.7: Illustration of two modules of NRPS including adenylation (A) domains that activate the amino acid for tethering to the thiolation (T) domains (phosphopantetheinyl linker shown in c) and the condensation (C) domain. The activated T-linked amino acid is bound to the growing peptide by the C domain. The C domain catalyzes peptide bond formation and is specific for activated amino acids of correct stereochemistry and so acts as a proofreading step to ensure only correct peptides are made. Seven modules are required to assemble the heptapeptide backbone of GPAs (8, 67).

The A domain acts to recognize a specific amino acid in an eight amino acid recognition pocket and adenylates the carboxyl group of the residue using ATP (24). The recognition pocket sequences of several A domains and their specificities have been reported, allowing the identity of the recognized residue to be inferred (68).

The activated amino acid becomes covalently bound to the enzyme via a phosphopantetheine (P-pant) linker extending from a conserved serine residue in the T domain, resulting in the release of AMP. The prosthetic P-pant group is added to the T domain by another enzyme called phosphopantetheinyl transferase (37, 61).

The Te (thioesterase) domain in the seventh module acts to release the completed peptide by hydrolyzing the thioester bond via a conserved serine residue in a non-specific manner (62). Epimerization domains can epimerize the most recently added L-amino acids to their D isomer, presumably by using an active site histidine residue to deprotonate the amino group, allowing racemization of the residue (69). Since the following C domain is specific for the correct stereoisomer, only the correct residue is added into the growing peptide (42).

The A47934 cluster, includes 4 NRPS genes that make up the seven modules required to assemble the backbone (StaA for modules 1 (HPG) and 2 (Tyr), StaB for module 3 (DHPG), StaC has modules 4 (HPG), 5 (HPG) and 6 (OH-Tyr), and StaD contains module 7 (DHPG)).

1.1.3 – GPA backbone cross-linking

The cross-links in A47934 are predicted to be formed by 4 P450 monooxygenases encoded by *staF*, *staG*, *staH* and *staJ*. In each GPA biosynthetic cluster sequenced to date, there is a distinct P450 for each cross-link and appropriately, there are four cross-links in A47934 (21, 54, 57, 65, 66, 71). Gene inactivation studies in *Amycolatopsis mediterranei* (balhimycin producer) showed that OxyA (StaF orthologue) forms the ether cross-link between residues OH-Tyr_2 and HPG_4, OxyB (StaH orthologue) catalyzes the HPG_4 to OH-Tyr_6 ether cross-linkage and OxyC (StaJ orthologue) performs the HPG_5 to $DHPG_7$ carbon-carbon cross-link (10, 11). The deletion mutants made in that study and the GPA intermediates isolated from each are shown in figure 1.8 below.

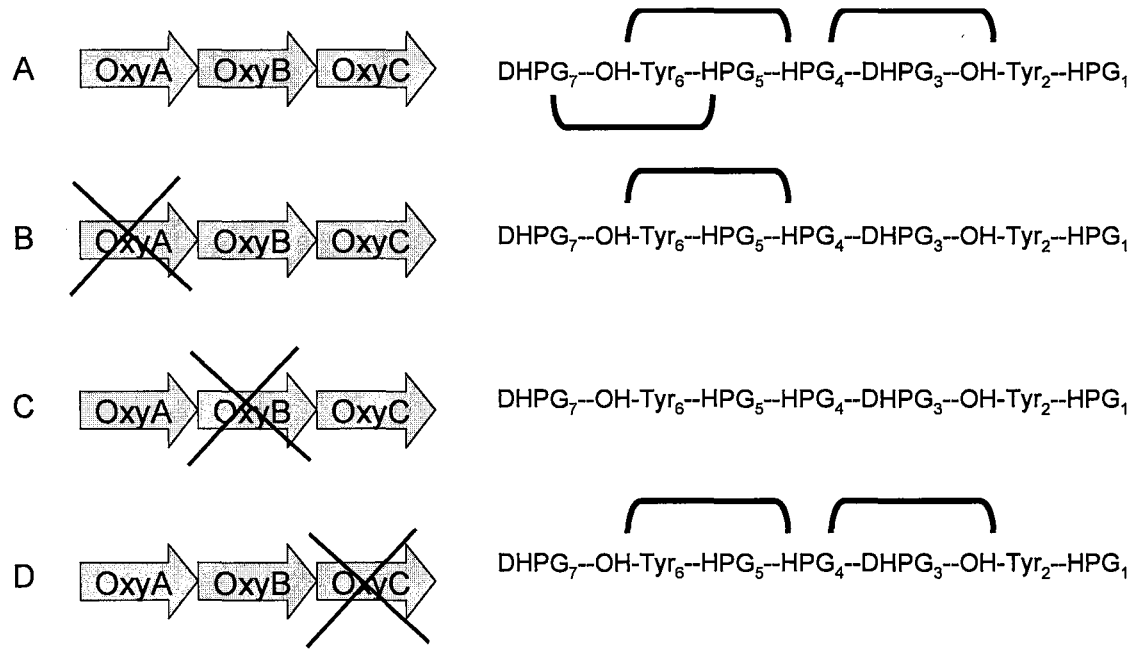


Figure 1.8- Summary of inactivation mutants created for studying cross-linking in the balhimycin producer and the semi-cross-linked intermediates produced by each. A) wild type strain performs all 3 cross-links B) *oxyA* mutant produces only singly cross-linked GPA intermediates C) *oxyB* mutant produces only linear peptide D) *oxyC* mutant performs 2 cross-links. These observations indicate the obligate cross-linking order OxyB→OxyA→OxyC, analogous to StaH→StaF→StaJ in the A47934 cluster. Figure adapted from reference (10).

The A47934 P450 that lacks an orthologue in *A. mediterranei*, StaG, is expected to form the HPG_1 and $DHPG_3$ cross-link not found in the vancomycin class GPAs. The presence of this additional P450-mediated cross-link confounds the cross-linking order in A47934 as well as any inferences about substrate specificity in teicoplanin-class GPA cross-linking. The crystal structures of OxyB and OxyC have been reported but *in vitro* activity has been difficult to generate.

1.1.4-Backbone Modification in A47934

A47934 has several modifications including three chlorinations and a sulphation. The chlorinations of Tyr_2 and OH-Tyr_6 are thought to be performed by StaI based on 88% sequence similarity with BhaA (balhimycin producer) and CepH (chloroeremomycin producer). The halogenation activity of BhaA has been characterized (58). The remaining non-heme halogenase in the A47934 cluster, StaK, is therefore predicted to chlorinate HPG_5.

The sulphonyl group at HPG_1 is added by StaL, an enzyme with high homology to other sulfotransferases and has been shown to be able to sulphonylate desulfo-A47934, teicoplanin and teicoplanin aglycone *in vitro* to generate novel antibiotics. StaL is a 3'-phosphoadenosine 5'-phosphosulphate (PAPS)-dependent sulfotransferase (36).

Although A47934 harbours only these modifications, other GPAs contain a myriad of others. Many natural product GPAs are glycosylated and some are lipidated. Figure 1.9 shows the backbone structure of both vancomycin and teicoplanin class GPAs and summarizes some of the known modifications of natural product GPAs, as well as oritavancin, a second generation, semi-synthetic GPA that will be discussed further in a later section.

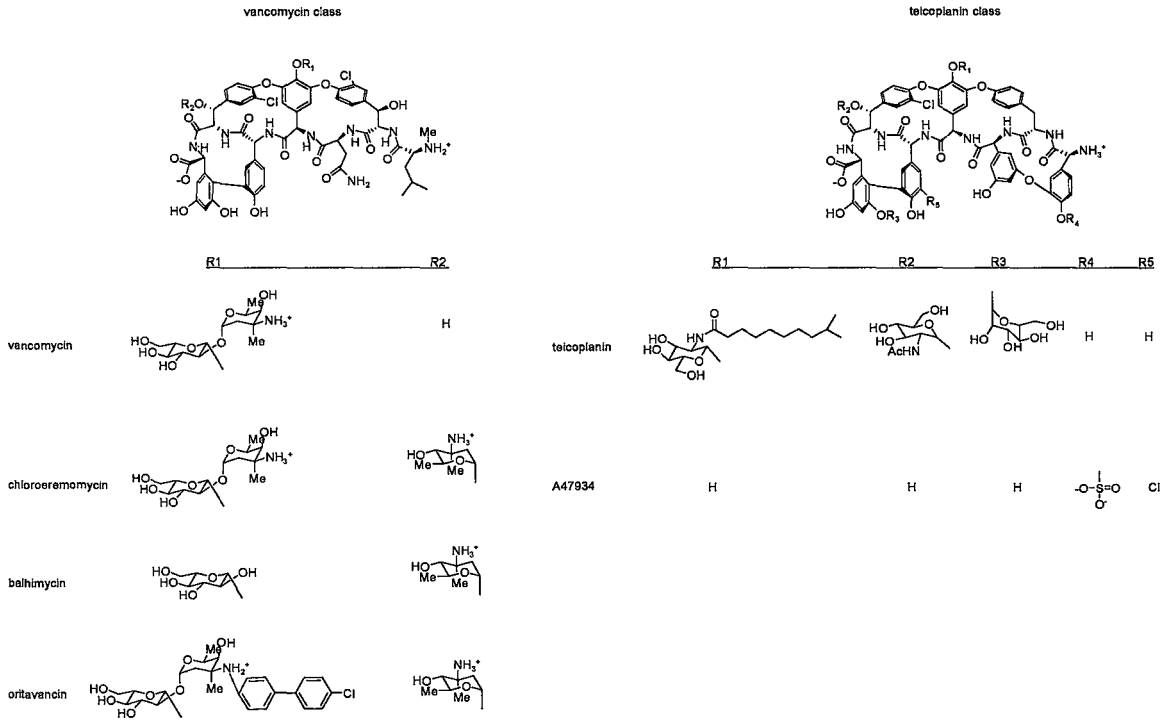


Figure 1.9 – Summary of backbone structures of vancomycin and teicoplanin class GPAs as well as some known combinations of backbone modification illustrating the diversity of modifications known (5, 57).

1.2 - GPA Mode of Action

Glycopeptide antibiotics exhibit their antimicrobial action due to a network of five hydrogen bonds between the rigid cross-linked molecule and the growing peptidoglycan layer present in most bacteria. The peptidoglycan layer is a meshwork polymer of sugars and peptides that give structural rigidity to the bacterial cell. The basic unit of peptidoglycan consists of N-acetylglucosamine and N-acetylmuramic acid with a pentapeptide linker extending from the latter. The pentapeptide usually consists of L-Ala-D-Glu-L-Lys-D-Ala-D-Ala with cross-linking between the monomers by a pentaglycine bridge that links a D-Ala of one monomer to the L-Lys of another. This transpeptidation, along with transglycosylation between the sugar residues give the cell rigidity and resistance to osmotic stress. These extracellular reactions of peptidoglycan biosynthesis are illustrated in figure 1.10 below.

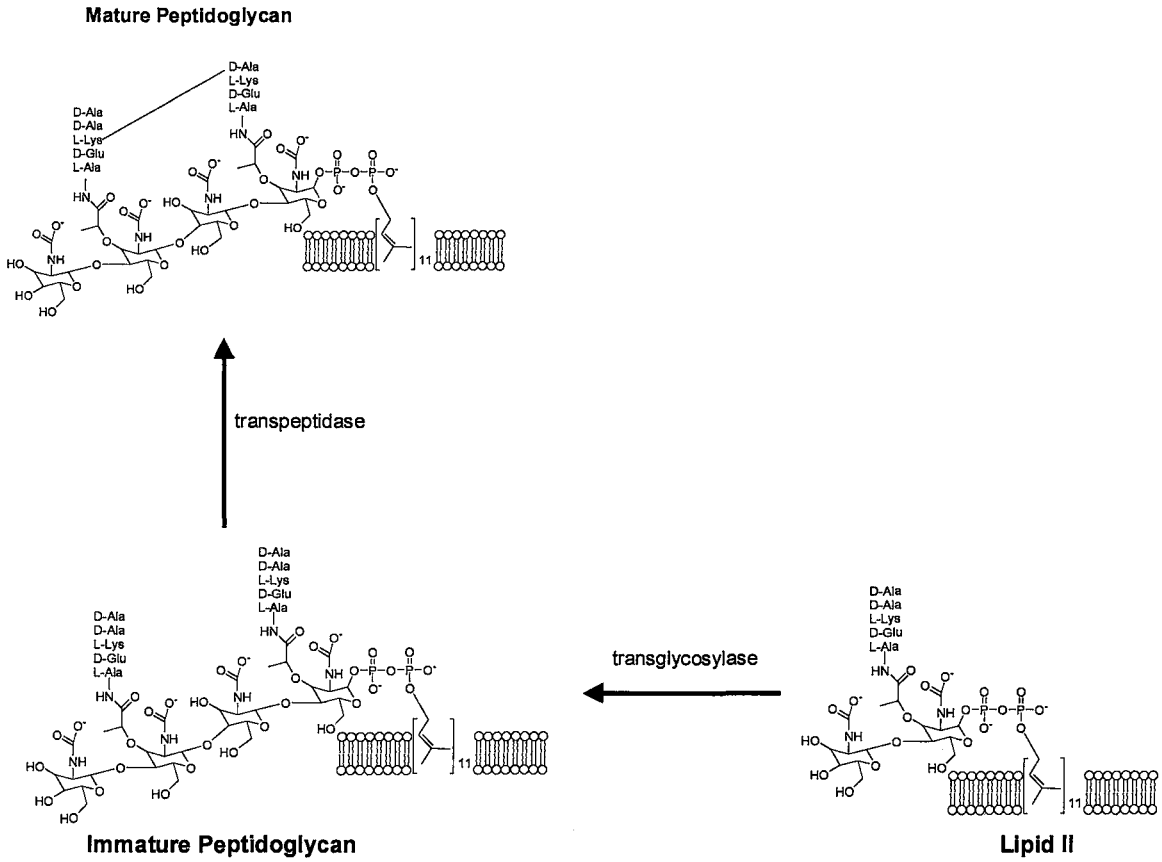


Figure 1.10– Summary of the final stages of peptidoglycan maturation. Transglycosylation of sugars residues and transpeptidation of pentapeptide chains give peptidoglycan structural rigidity, which in turn confers resistance to osmotic stress for the cell (20).

GPAs bind with high affinity to the D-Alanyl-D-Alanine (D-Ala-D-Ala) terminus before cross-linking occurs to disrupt transpeptidation by sequestering the D-Ala-A-Ala cross-linking locus and by steric hindrance to transglycosylation and transglycosylation (4). The hydrogen bonding between vancomycin and the D-Ala-D-Ala target is illustrated in figure 1.11 below.

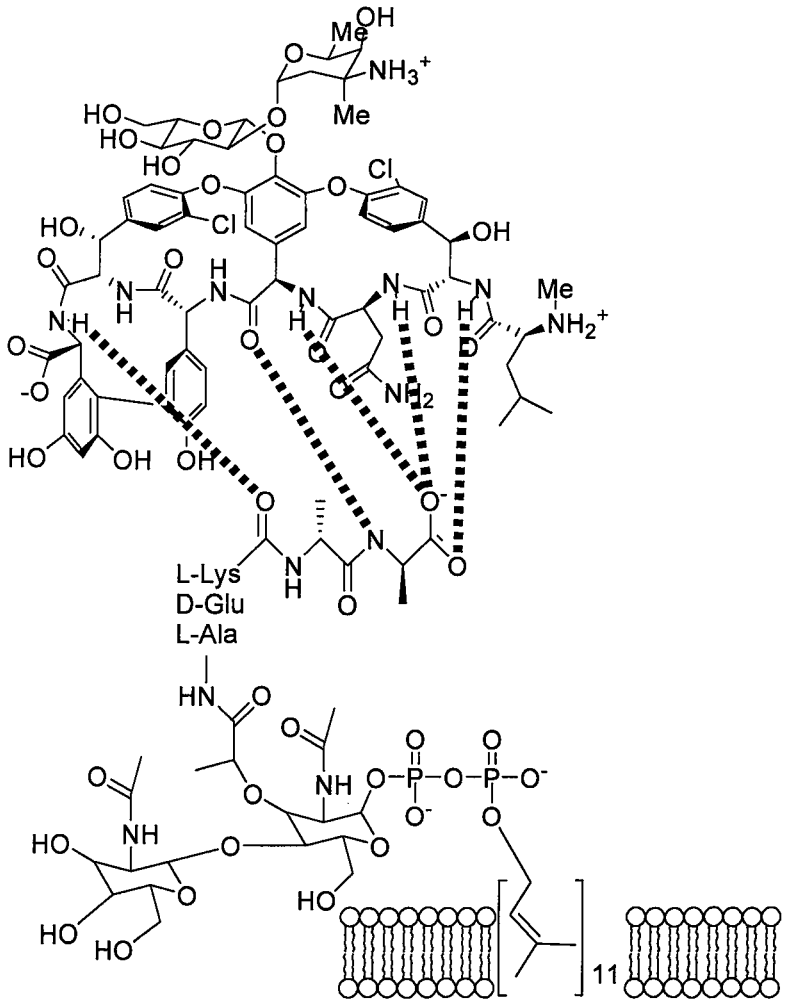


Figure 1.11– Structure of vancomycin bound to D-ala-D-ala terminus of growing peptidoglycan chain highlighting the 5 hydrogen bonds (bold, dashed) responsible for high affinity binding. This binding sequesters the substrate for transpeptidase and hinders transglycosylation, ultimately leaving the cell susceptible to osmotic stress (4).

Other factors may influence the efficacy of different GPAs. It has been reported that carbohydrate substituted GPAs can inhibit the transglycosylase enzyme directly, conferring multivalent activity to these compounds (26). Some glycopeptides (such as the vancomycin class GPA chloroeremomycin) can form dimers, while some GPAs (such as teicoplanin) have fatty acid functionality that may allow them to associate closely with each other and the cell membrane. Both characteristics have been reported to increase

affinity for peptidoglycan and antibiotic activity by bringing the GPA closer to its target (7, 27).

1.3 - Resistance to Glycopeptide Antibiotics

Resistance to GPAs is of growing concern, as they are often the antibiotic of choice or last resort for multiple antibiotic resistant infections. Resistance to GPAs first presented itself clinically in VRE during the 1980s. Since then, many resistance mechanisms have been identified for various potentially pathogenic organisms. Most of the resistance mechanisms result in a change in cell wall composition to render GPAs unable to bind peptidoglycan with high enough affinity to be clinically useful. Table 2 compares three resistance phenotypes.

Table 2- Comparison of Three Vancomycin Resistance Phenotypes

(summarized from references (39, 53))

Phenotype	Peptidoglycan terminus	MIC[a] (μg/mL)	Source	Induction
VanA	D-Ala-D-Lac	Vanc (>1000) Teic (500)	Acquired e.g. Tn 1546	Inducible
VanB	D-Ala-D-Lac	Vanc (32)	Acquired e.g. Tn 1547	Inducible
VanC	D-Ala-D-Ser	Vanc(2-32)	Intrinsic	Constitutive and Inducible
VSE[b]	D-Ala-D-Ala	Vanc (0.5-2) Teic (0.25-2)	Wild type	

[a]MIC: Minimal Inhibitory Concentration; [b]VSE: Vancomycin Sensitive Enterococci

The first GPA resistance cluster to be characterized was VanA. In this cluster, *vanH* encodes a α-keto reductase that converts pyruvate to D-lactate (D-Lac) and the *vanA* gene encodes a D-Ala-D-Lac ligase that has only low D-Ala-D-Ala activity (15, 16, 45). The *vanX* gene product acts as a Zn-dependent metallodipeptidase that cleaves D-Ala-D-Ala but leaves D-Ala-D-Lac intact (77). Figure 1.12 below outlines the activities of these three enzymes.

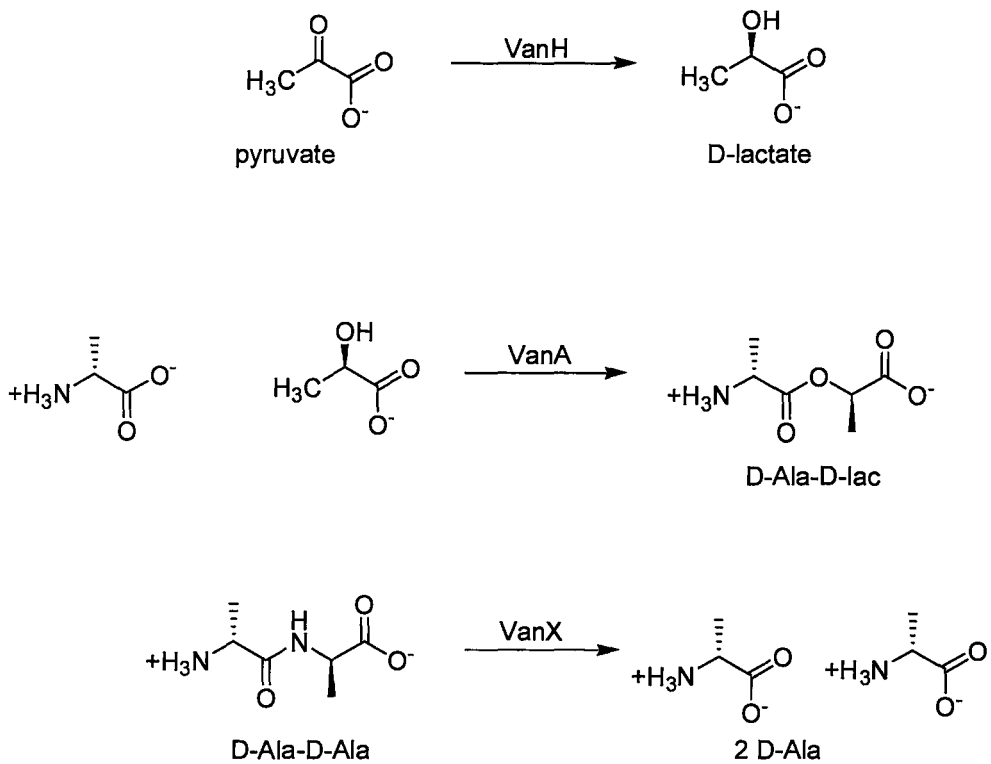


Figure 1.12– Summary of VanH, VanA and VanX activities, the three enzymes responsible for altering the peptidoglycan terminus from D-Ala-D-Ala to D-Ala-D-Lac, conferring resistance to GPAs (15, 16, 45, 77).

Through the action of these three enzymes, D-Ala-D-Lac becomes more abundant and is incorporated into the growing peptidoglycan layer. The incorporation of lactate changes a peptide bond to an ether bond, which eliminates a hydrogen bond between GPAs and peptidoglycan. This bond loss reduces GPA affinity for peptidoglycan 1000-fold, making them clinically useless.

In many cases, the *vanHAX* genes are inducible and controlled by a two component regulatory system. The *vanS* gene encodes a sensor kinase that autophosphorylates a conserved histidine residue in response to a GPA-induced signal and in turn phosphorylates the *vanR* gene product on a conserved aspartate residue.

VanR in its phosphorylated form acts as a transcriptional regulator for the other genes in the resistance cluster (3, 76). This is shown schematically in figure 1.13.

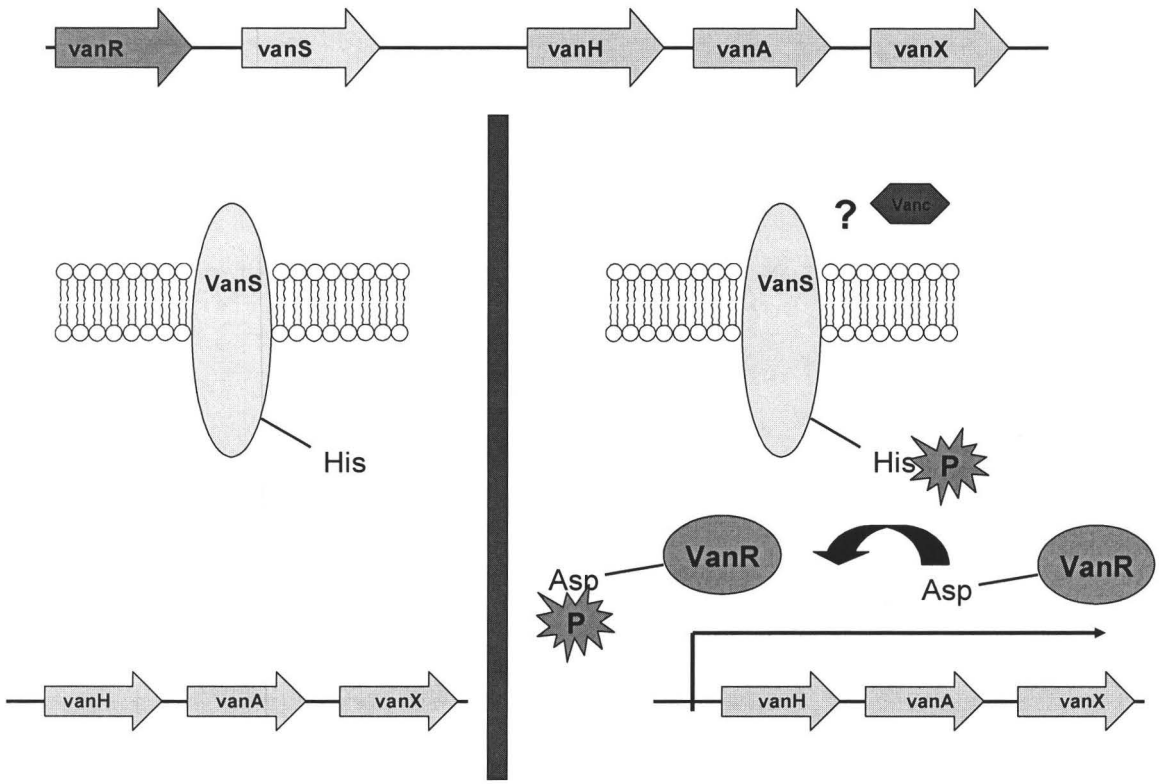


Figure 1.13– In the absence of vancomycin (left), both VanS and VanR are dephosphorylated and the *vanHAX* genes are off. In the presence of vancomycin, an uncharacterized signal leads to VanS autophosphorylation and subsequent phosphorylation of VanR, leading to activation of *vanHAX* genes and the resistance phenotype (3).

The VanC phenotype is slightly different from the VanA and VanB phenotypes. Instead of a ligase that produces D-Ala-D-Lac, the VanC cluster encodes a ligase that makes D-Ala-D-Ser. The result is the same, the GPA binding affinity is reduced and the antibiotic becomes clinically ineffective against the organism. This cluster also contains a VanRS two component regulatory system (2).

1.4 - Overcoming GPA resistance

There have been several different approaches to evade GPA resistance mechanisms discussed in the literature. Differential modification of the backbone, altered backbone (where hydrogen bonding occurs) and targeting the resistance mechanism directly are three options that will be discussed in this section.

1.4.1 Differential Backbone Modification in GPAs

The various glycosylations, lipidations and halogenations of GPAs with similar backbones immediately raises questions about the specificity of the tailoring enzymes responsible for adding these moieties. Much research in this area has shown that the glycosyltransferase enzymes of various GPA clusters can modify the GPAs produced in other species. In fact, using aglycone intermediates of both teicoplanin and vancomycin class GPAs, a diverse library of differentially glycosylated GPAs can be generated (72). Although these novel molecules have a range of activities, none have become clinically useful against VRE or VRSA. The approach of genetically modified organisms producing novel GPAs is appealing as the total synthesis of GPAs is a complex process with low yields and accompanied high cost. As our knowledge of GPA modification enzymes grows, and the number of available genes increases, this area will continue to be of great interest.

The growing diversity of natural and genetically engineered GPAs has led to another approach to GPA development. Semi-synthetic derivatives of natural products have found clinical use against infections caused by vancomycin resistant bacteria. In 1989 Eli Lilly Research Laboratories tested the ability of many semi-synthetic GPAs

(mainly synthetic groups added to glycosyl moieties of vancomycin) to retain activity against resistant organisms. One compound, chlorobiphenyl vancomycin, indeed met these expectations (47-49). The structures of chlorobiphenyl vancomycin as well as Oritavancin, a second generation GPA in clinical trials are shown in figure 1.14 below.

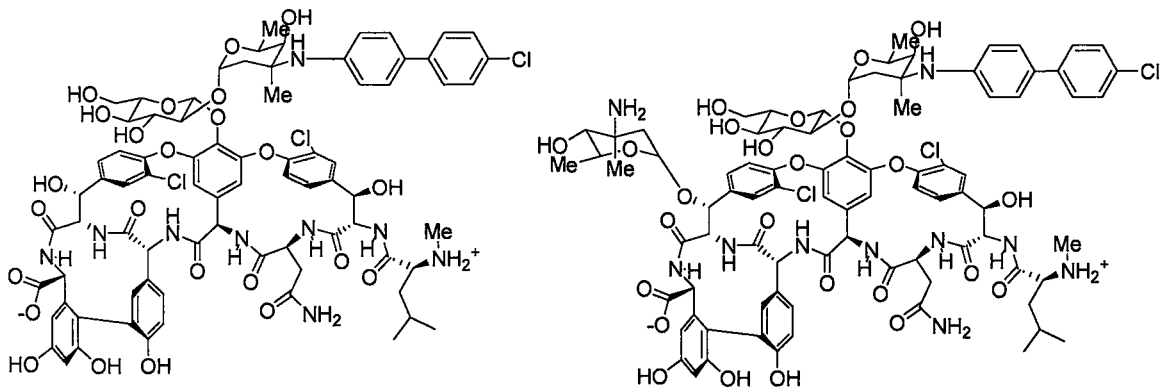


Figure 1.14: Structures of chlorobiphenyl vancomycin (left) and InterMune's Oritavancin (right), a chloroeremomycin derivative currently in clinical trials (5, 49).

One prominent theory as to how the chlorobiphenyl group gives rise to increased activity is that this group interacts directly with the lipid bilayer, in turn converting the association between the compound and the target to an intramolecular interaction, making up for the hydrogen bond loss.

Elegant experiments by Kahne *et al.* showed that this was likely not the only mechanism for the activity observed against VRE. By eliminating the N-terminal Leucine residue, the D-Ala-D-Ala binding pocket is damaged; the chlorobiphenyl compound remained active while normal "damaged vancomycin" did not. In addition, a chlorobiphenyl compound that was not a GPA at all had some activity against VRE (Table 3) (20, 26). The structures of damaged vancomycin and the non-GPA chlorobiphenyl compound are shown below.

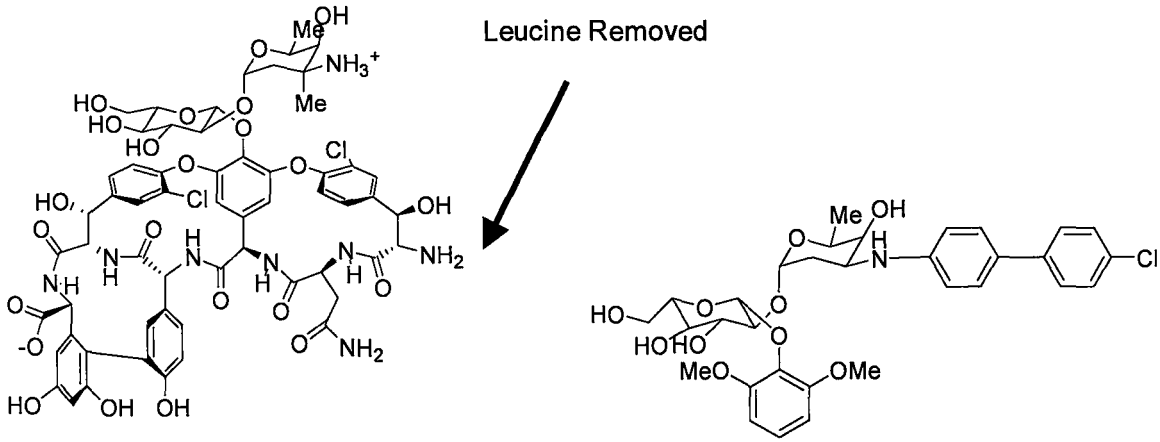


Figure 1.15- Left: Damaged (des-leucyl) vancomycin, Right: non-GPA chlorobiphenyl compound shown to have modest antimicrobial activity against VRE (20).

Table 3 - MICs of vancomycin derivatives Against Enterococci (20, 26, 47)

MIC μg/mL

	E. faecium		*E. faecalis*	
	sensitive	resistant	sensitive	resistant
Vancomycin	1	2048	4	2048
Chlorobiphenyl Vancomycin	0.03	16	0.025	16
Damaged Vancomycin	No activity	No activity	No activity	No activity
Damaged Chlorobiphenyl Vancomycin	10	20	40	80
Chlorobiphenyl Disaccharide	128	128	128	128

This observation inspired another set of experiments that measured accumulation of peptidoglycan intermediates in a permeabilized *Escherichia coli* strain. In *E. coli*, peptidoglycan is assembled sequentially such that inhibition of transpeptidation leads to accumulation of immature peptidoglycan, while inhibition of transglycosylases results in lipid II accumulation. These experiments showed that in the presence of natural vancomycin, immature peptidoglycan was the predominant intermediate isolated, while with chlorobiphenyl vancomycin treatment, lipid II was the major intermediate detected

(20). These results suggest that vancomycin primarily inhibits transpeptidation, while the chlorobiphenyl derivative inhibits the earlier transglycosylation step (20). Transglycosylation inhibition has been directly verified *in vitro* using *S. aureus* PBP2, the primary bifunctional transglycosylase and transpeptidase in the bacterium (41). Eli Lilly later found that chlorobiphenyl chloroeremomycin (similar to vancomycin with additional glycosyl group) displayed better antibiotic activity. This compound, (Oritavancin, now licensed to InterMune, Figure 1.14) is in phase III clinical trials (5).

Yet another potential mechanism of action of GPAs has been proposed based on observations that Telavancin (a semi-synthetic vancomycin derivative, Figure 1.16) causes loss of membrane integrity and loss of ATP and membrane potential, while vancomycin does not. These experiments involved the use of fluorescent dyes to indirectly measure these criteria and it was shown that the disruption of membrane integrity correlated with decreased cell viability (29).

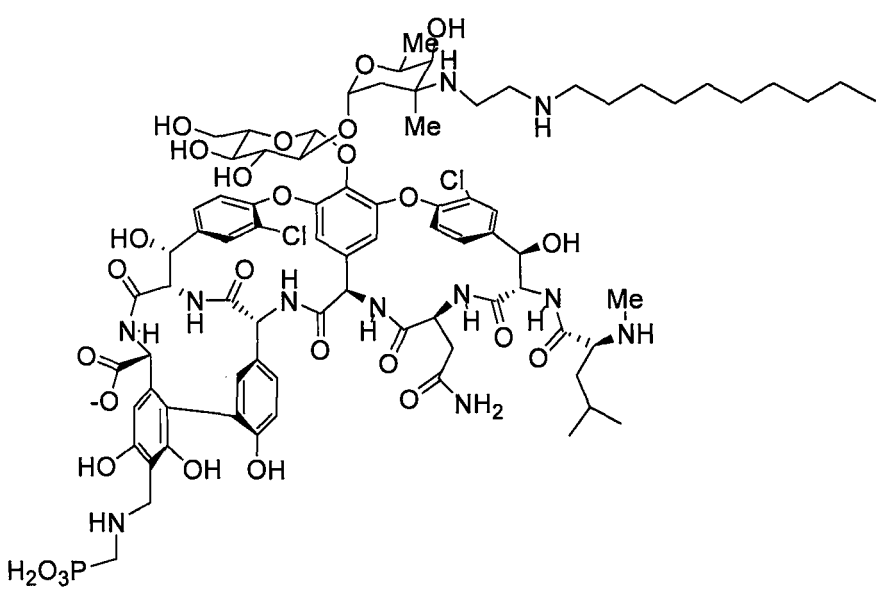
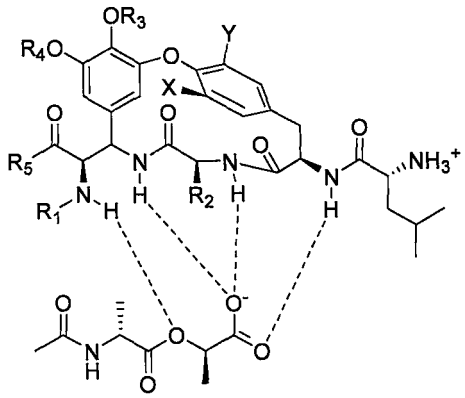


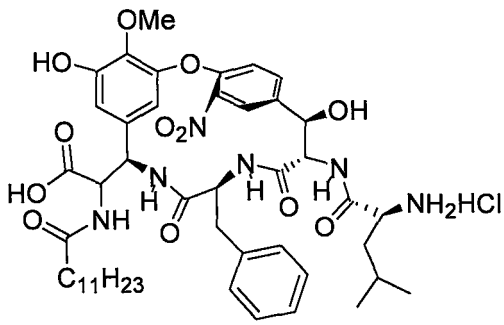
Figure 1.16- Structure of Telavancin (Theravance), a vancomycin derivative active again VRE and shown to affect membrane integrity. Modified from reference (29).

1.4.2-Altered D-Ala-D-Ala Binding Pocket in GPAs

The D-Ala-D-Lac terminating peptidoglycan results in the loss of a hydrogen bond between GPAs and their target; in addition, electronic repulsion may add to the decrease in affinity observed. The atoms of the GPA that form the peptide binding pocket are in the peptide backbone. Theories have been proposed that if the carbonyl group that has the potential to generate electronic repulsion were changed to a slightly positively charged group, activity against resistant strains could be restored.

One group has designed compounds consisting only of the peptide binding pocket modified to contain an amine at this position and found that these compounds do indeed have activity against VRE (and appropriately, limited activity against susceptible strains) (44). The total synthesis of these complex compounds is not amenable to mass-production and therefore it is desirable to manipulate biosynthetic machinery to produce these novel compounds (Figure 1.17) (44).





MIC µg/mL

E. faecium		*E. faecalis*		*S. aureus*
sensitive	resistant (VanA)	sensitive	resistant (VanB)	
128	8	4	4	64

Figure 1.17- The general structure of engineered compounds with modified backbone that show activity against VRE, highlighting how they are predicted to bind D-Ala-D-Lac (top left, X and Y are sites of variable modification); Structure of compound with highest activity (top right); MIC of best compound against VRE and *S. aureus* (bottom table). Adapted from reference (44).

NRPSs have been engineered to have altered specificity and many different residues can be incorporated into a peptide using these modified enzymes under ideal conditions. One hindrance is the fact that the C domain has proofreading activity, the specificity of which requires more study. Additionally, the specificity of the cross-linking P450 enzymes is largely unknown. P450-mediated cross-linking in A47934, specifically substrate specificity is the focus of the primary study in this report and is discussed in section 2.

1.4.3-GPA Resistance Enzymes as Drug Targets

Significant efforts have been made to develop inhibitors of the VanHAX enzymes in order to prevent the resistance phenotype. Drugs that attain this goal would be invaluable in combination therapy for infection due to resistant organisms. Although some inhibitors have been reported, none have come to clinical use to date (16).

Inhibition of the two component regulatory system directly may be possible with kinase inhibitors, of which a diverse library exists. This approach may not be feasible in human treatment as these drugs would have multiple metabolic side effects. It has been proposed that blocking VanS autophosphorylation would be futile as endogenous kinase activity could still activate VanR. A counterpoint to this suggestion is that *vanS* deletion mutants lose the resistance phenotype (73). Finding molecules that can down-regulate VanHAX expression and activity by interfering with the external signal leading to VanS autophosphorylation is the second focus of this study. The first step in this work was to clarify the external signal by finding the membrane proteins that specifically recognize and bind GPAs and activate VanS autophosphorylation. This work is described in section 3.

1.5 – Research Objectives

Two studies are discussed in this thesis, both relating to different approaches to overcoming GPA resistance. One section deals with generating novel GPAs while the other focuses on inhibiting the resistance mechanism itself in hopes of finding leads for combination therapy against GPA resistant species.

Chapter 2 discusses the role of P450 monooxygenases in the cross-linking of GPAs. The cross-links are required to give the molecules structural rigidity and form the D-ala-D-ala binding pocket. In light of the fact that synthetic GPA-like molecules show activity again resistant strains, it is attractive to genetically modify natural GPA producers to create these difficult to synthesize compounds. Both the NRPSs and the cross-linking P450 monooxygenases need to be better understood to achieve this goal. The specific goals of this part of the project are to clone, express, purify and generate in vitro activity for all P450s in the A47934 biosynthetic cluster in hopes of better understanding these enzymes and their substrate specificity in hope of someday being able to alter these enzymes such that they can cross-link novel backbones and thus generate novel antibiotics which may be useful in treating infection due to GPA resistant organisms.

Chapter 3 is an investigation of the molecular events leading to VanS autophosphorylation and VanHAX expression. Specifically, the goal is to identify vancomycin binding proteins in resistant and susceptible species in hopes of finding those responsible for GPA recognition and activation of VanS. This is especially important in GPA producing bacteria. The ultimate goal is to leverage this information to find compounds that can inhibit the resistance response to GPA presence.

Section 2: Exploration of P450-mediated cross-linking in glycopeptide antibiotic biosynthesis

First of 2 studies

2.0 - Introduction to P450 Mediated GPA backbone Cross-linking in A47934

There are four intra-strand cross-links between residues in A47934. These cross-links give the molecule a rigid structure and form the peptide binding pocket which participates in the five hydrogen bonds with D-ala-D-ala that lead to the high affinity between drug and target. There are four P450 monooxygenase enzymes encoded in the A47934 biosynthetic cluster (StaF, StaG, StaH and StaJ) (57). Based on homology to enzymes in the balhimycin producer it is predicted these enzymes work in the order StaH (HPG_4 and OH-Tyr_6) followed by StaF (OH-Tyr_2 and HPG_4) followed by StaJ (HPG_5 to $DHPG_7$). The additional cross-link is predicted to be performed by StaG (HPG_1 and $DHPG_3$) although the order is unknown (10, 11, 57). The complicated nature of the cross-linking order in teicoplanin class GPAs is illustrated in figure 2.1 below, showing only one possible initial substrate, the linear heptapeptide.

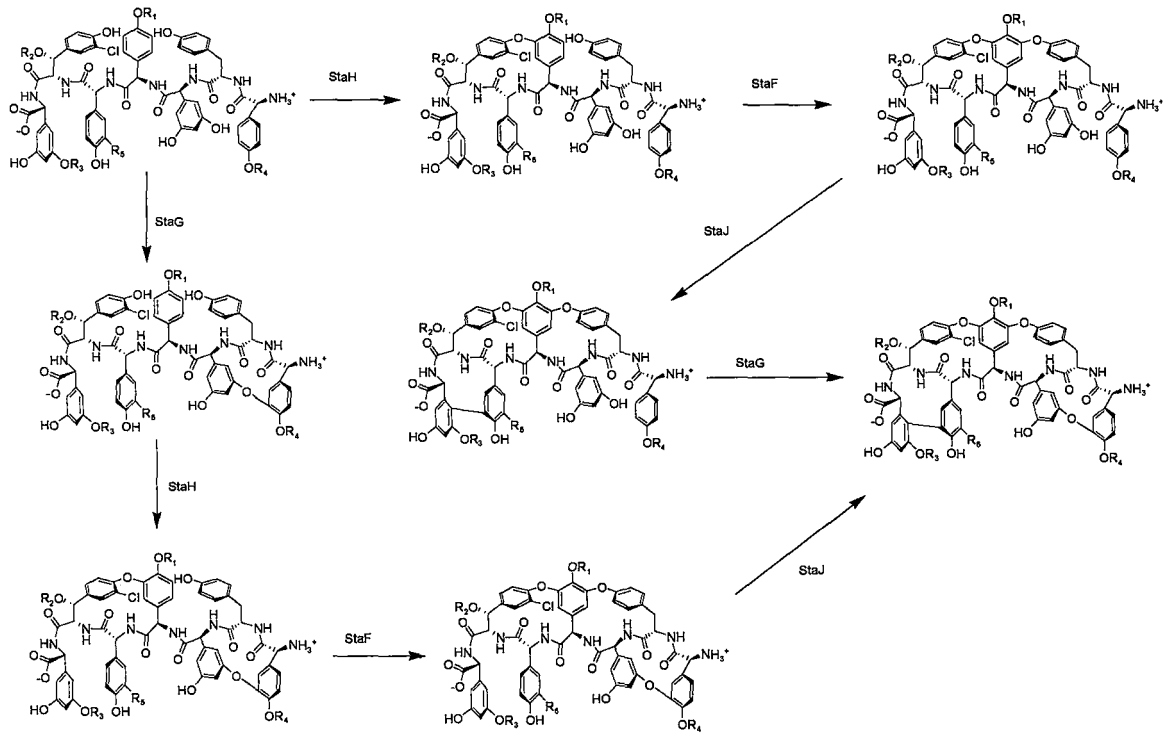


Figure 2.1- Two possible scenarios for the cross-linking reactions in the teicoplanin-class GPAs showing the structures of the intermediates using free linear heptapeptide as the substrate for the first cross-linking P450. Because the order of cross-linking in teicoplanin-class GPAs is unknown, it is possible there is no conservation from the vancomycin-class order.

2.1 - Cytochrome P450 monooxygenase activity and mechanism

Cytochrome P450 monooxygenases (P450s) are a large family of heme-containing proteins involved in the oxygenation of diverse substrates, often by hydroxylation. The literature surrounding P450s is extensive and dates to before their naming in the 1960's (51, 52). The enzymes require the input of two electrons, one at a time, to drive a catalytic cycle as highlighted in figure 2.2 (34, 50, 75).

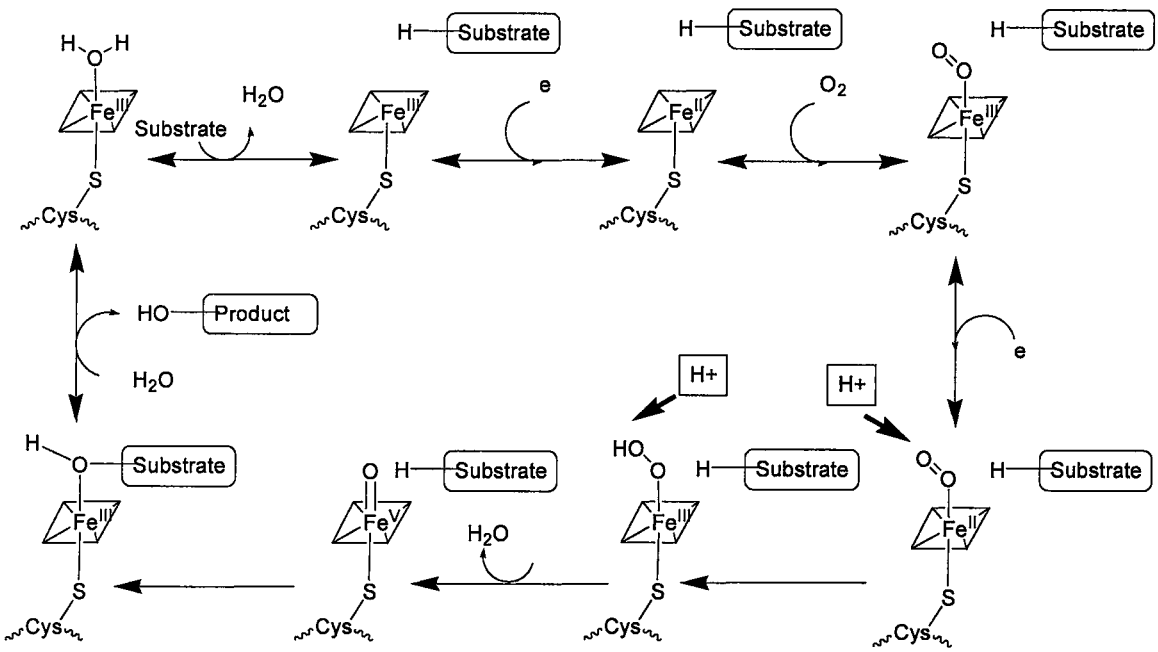
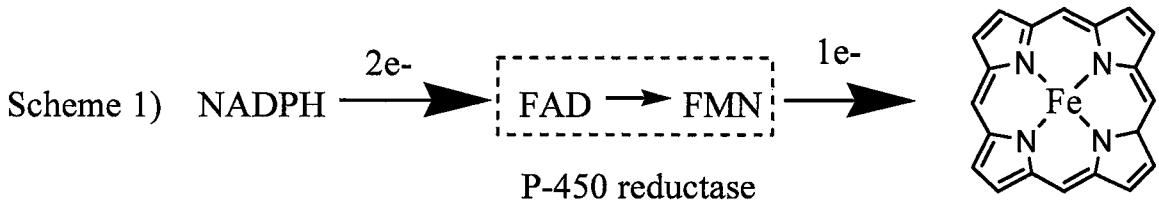
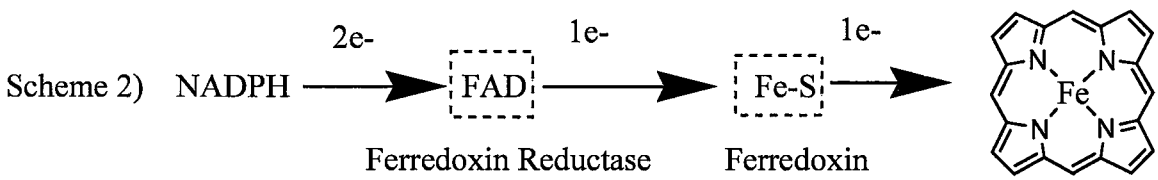


Figure 2.2- Catalytic cycle of P450 monooxygenases showing electrons being introduced one at a time to generate the highly reactive iron-oxo (Fe^{V}=O)intermediate. Modified from reference (75).

These electrons are donated by NAD(P)H as a pair and are passed through flavin-containing intermediates to be transferred to the P450 one at a time. In mammalian systems, the electron transferring intermediate is a single protein containing both FAD and FMN (P450 reductase, scheme 1) (28).



In bacterial systems, there are generally two intermediates, a FAD-containing ferredoxin reductase and an iron-sulphur protein (ferredoxin, scheme 2).



The electrons are used to generate a highly reactive iron-oxo intermediate that is capable of hydroxylating the substrate (see figure 2.2) (17, 50). It has been proposed for the cross-linking P450s that the reactive intermediates catalyze the formation of radicals, which then rearrange to form the cross-linking bond (Figure 2.3) (31).

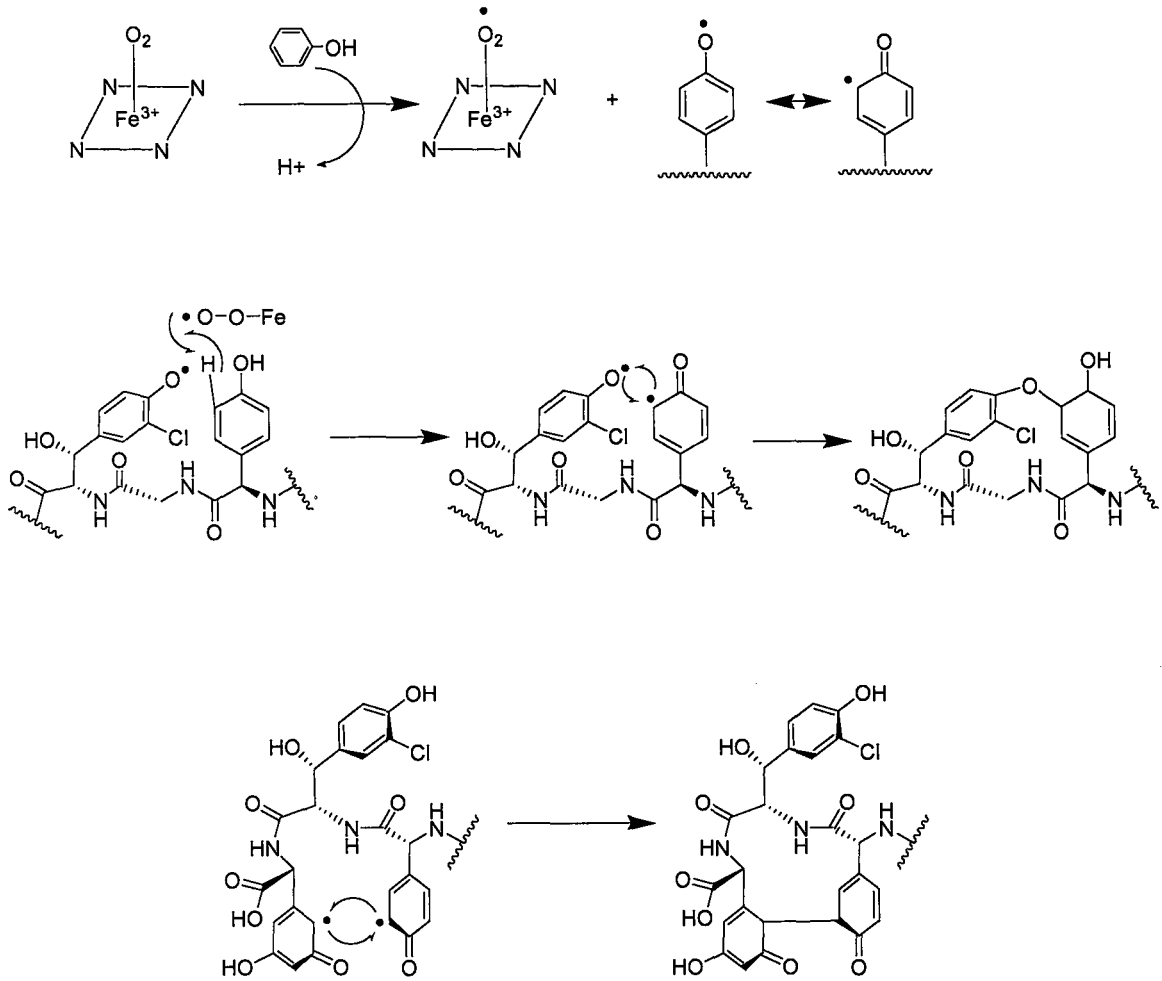


Figure 2.3- Proposed radical mechanism for P450-mediated cross-linking in GPAs. P450s commonly hydroxylate substrates but these mechanisms explain how they may mediate GPA backbone cross-linking. Modified from reference (31).

It was recently reported in the literature that *in vitro* activity has been generated with OxyB and a hexapeptide still tethered to a recombinant segment of the T domain of the sixth module of the NRPS. The group cloned a small segment of the NRPS surrounding the conserved Ser residue that binds the phosphopantetheinyl linker in the sixth module. The linear hexapeptide was synthesized and the C-terminus was modified with a phenol group via a thiol bond, which was subsequently replaced by CoA. This compound was used as a substrate for a phosphopantetheinyl transferase from *Bacillus*

subtilis, thus tethering the synthetic hexapeptide to the cloned NRPS apo-protein. This substrate was reacted with purified OxyB, spinach ferredoxin and ferredoxin reductase and a monocyclic product was produced (complete reaction scheme shown in Figure 2.4) (9, 79).

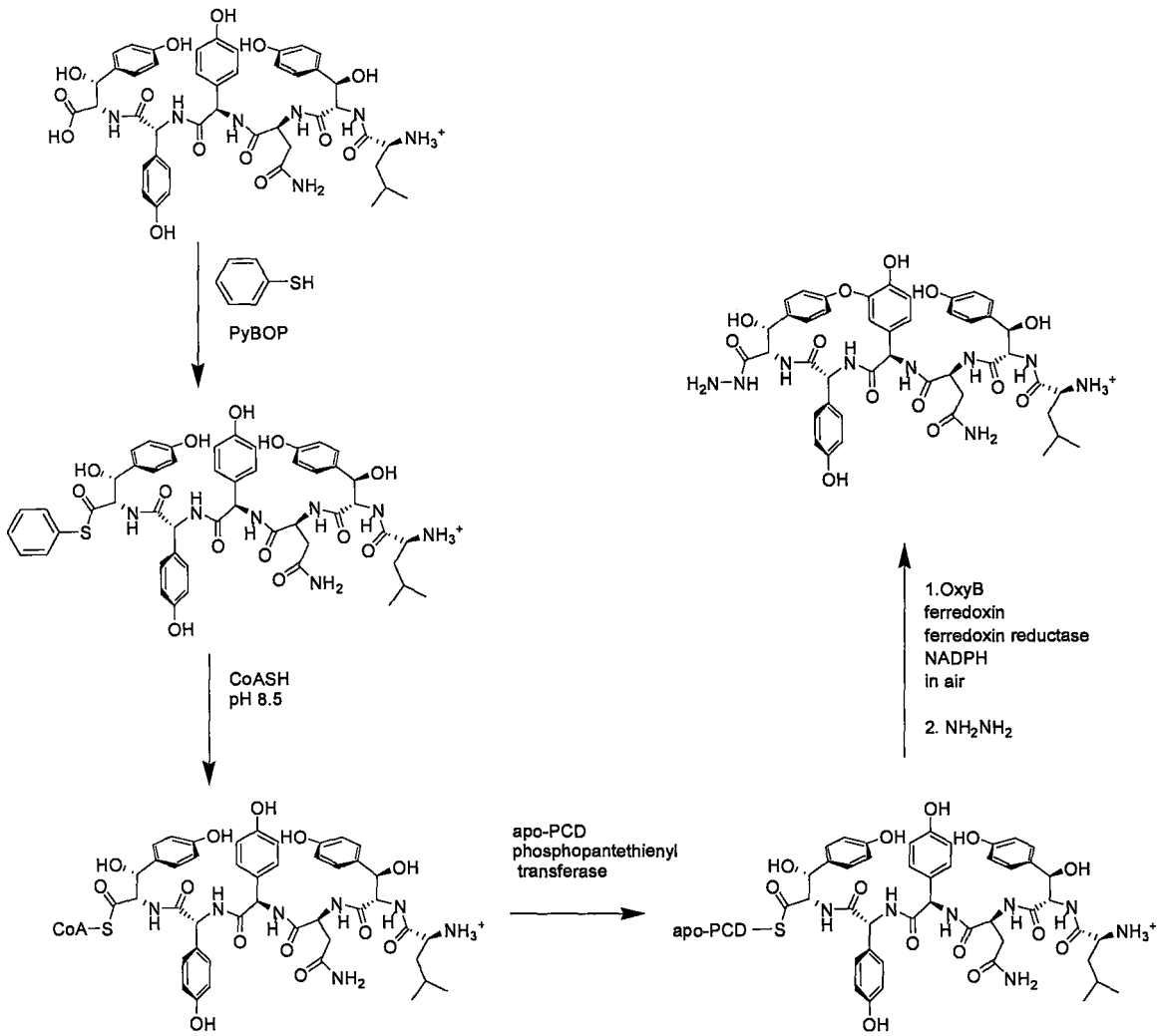


Figure 2.4- Reaction scheme to generate monocyclic hexapeptide using OxyB. The hexapeptide (top left) was synthesized and modified to harbour a CoA moiety at the C-terminus. This was used as a substrate for a phosphopantetheinyl transferase to transfer the hexapeptide to a cloned segment of the appropriate NRPS. This tethered hexapeptide proved to be a substrate for OxyB, the first cross-linking P450 in vancomycin class-GPAs. Summarized from reference (79).

2.2 - Goals of the A47934 cross-linking study

At the inception of this project, virtually nothing was known about the substrate specificity of the cross-linking P450 monooxygenases. The goal of this study was to generate in vitro activity for the P450s in the A47934 cluster. In light of the fact that attempts to generate in vitro activity with purified OxyB had failed with commercially available spinach ferredoxin and ferredoxin reductase, we postulated that either electron transport or initial substrate was incorrect (78). Our approach was to clone each of the P450s as well as ferredoxins and ferredoxin reductases from *Streptomyces coelicolor*, express and purify these proteins in order to generate activity with the predicted substrate, the linear heptapeptide substrate. We predicted the *S. coelicolor* enzymes would drive catalysis as they had been used to drive catalytic turnover of *Streptomyces griseolus* P450s (35, 40). This would allow us to clarify the substrates for each of the enzymes as well as the order in which the cross-links occur in teicoplanin class GPAs.

While this work was in progress the *in vitro* activity for OxyB was reported (79). As a result, this project was put aside to start a larger, more impactful project that would be a PhD-caliber research endeavor (next section). The final goal of this project was to create co-expression constructs that would allow expression of *S. coelicolor* ferredoxin / ferredoxin reductase as well as each of the P450s from the A47934 cluster as well as a P450 from *Steptomyces griseolus* and test the ability of *E. coli* harbouring each of these 5 constructs to convert 7-ethoxycoumarin to 7-hydroxycoumarin. In this experiment, the *S. griseolus* P450 would act as a positive control and I could comment on the substrate specificity of StaF, StaG, StaH and StaJ and show that my co-expression system was adequate to drive P450 catalytic cycles.

2.3 - Materials and Methods for cross-linking study

2.3.1 - Cloning of P450s, ferredoxins and ferredoxin reductases

Cloning strains of *E. coli* used were XL10 gold (Stratagene) and TOP10 (Invitrogen). Expression strains of *E. coli* used were BL21(DE3) and BL21(DE3)pLysS (both Stratagene). Recombinant DNA procedures were performed as described by Sambrook et. al. (60). Primers were obtained from MOBIX (McMaster University) with sequences as shown in Table 4.

Table 4 – Primers used to amplify target genes

Gene	Organism	Primers
staF P450	*S. toyocaensis*	F:AGCCATATGTTCGAGGAGATCAACGTCGTC R:AGCAAGCTTCGGGCTCGGTGTCGTATTC
stag P450	*S. toyocaensis*	F:AGC CAT ATG GCA CTT CCC TTG CCG CAC R:AGC AAG CTT GAA GCC CTG CCG GAG CGT GTG
staH P450	*S. toyocaensis*	F:AGCGCTAGCTTGAGTGGTGACGACCGGCC R:AGCAAGCTTATCTGATACCGCGGGAATACCTC
staJ P450	*S. toyocaensis*	F:GGGAATTCCATATGAGAAGAACGCTCTGCGATCC R:AGCAAGCTTTCGAACGTCTCTGCCGACATCTCT
sco0681 reductase	*S. coelicolor*	F:GGGAATTCCATATGCCCCGCCCTCTGC R:GGGAATTCAAGCTTGGATCCACTAGTTCAGGCGCCGCTCTCGC
sco2469 reductase	*S. coelicolor*	F:GGGAATTCCATATGGTGGTCGACGCGGATCAGACAT R:GGGAATTCAAGCTTGGATCCACTAGTCTATGCGACGAGGCTTTCGAGG
sco7117 reductase	*S. coelicolor*	F:GGGAATTCCATATGCCGCGTGCGAAGACG R:GGGAATTCAAGCTTGGATCCACTAGTTCACGCCTGGTCTCCCGTC
sco0773 ferredoxin	*S. coelicolor*	F:GGGAATTCCATATGCACATCGGCATCGACAAG R:GGGAATTCAAGCTTGGATCCACTAGTTCAGCCGACCCGCTCCG
sco1649 ferredoxin	*S. coelicolor*	F:GGGAATTCCATATGAGCGTGCAGCAGGAGGC R:GGGAATTCAAGCTTGGATCCACTAGTTCACTCTGAGTCCGGACCGTAGATC
sco3867 ferredoxin	*S. coelicolor*	F:GGGAATTCCATATGAGGATCTCCGTCGACCCC R:GGGAATTCAAGCTTGGATCCACTAGTTCAGCCCCGGACCCCTTC
sco5135 ferredoxin	*S. coelicolor*	F:GGGAATTCCATATGGTGACCTACGTCATCGCGCAG R:GGGAATTCAAGCTTGGATCCACTAGTTTACTGGTTCTGCGGCGGCA
sco7110 ferredoxin	*S. coelicolor*	F:GGGAATTCCATATGACTTACGTCATCGCACAGCCCT R:GGGAATTCAAGCTTGGATCCACTAGTTCAGGAGGGGAACCACAGATCC
sco7676 ferredoxin	*S. coelicolor*	F:GGGAATTCCATATGACCTTGGCAGGCCAGG R:GGGAATTCAAGCTTGGATCCACTAGTCTACCGAAGAGTGAGCGCTCCA
star flavoprotein	*S. toyocaensis*	F:GGGAATTCCATATGACGTCAACGACAGCGCAG R:GCGCCCAAGCTTGGATCCACTAGTTGGACGTCAGGAGGGAAGCG

F: forward primer
R: reverse primer

All genes were amplified as PCR (polymerase chain reaction) fragments and cloned into pET28a after digestion with *Nde* I and *Hind* III sites incorporated in the primers (*Nde* I from New England Biolabs, all other restriction enzymes used in this work were from Fermentas). Cloned gene sequences were verified by nucleotide sequencing (MOBIX, McMaster University). These constructs were transformed into chemically competent expression strains of *E. coli*.

2.3.2 - P450, ferredoxin and ferredoxin reductase protein expression

For protein expression, *E. coli* BL21(DE3) cells were grown in Luria broth (LB, Sigma) at 37 °C with shaking at 250 rpm to OD600 nm of 0.6 before adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Media were supplemented with 50 μg/mL kanamycin (BioShop Canada, Ltd.). Expression for 3 h at 37 °C was sufficient for over-expression of some reductases (visualized by sodium dodecyl sulphate polyacrylamide gel electrophoresis, SDS PAGE). For P450 over expression, 0.1 mM δ-aminolevulinic acid (a heme precursor to relieve metabolic stress of over-expressing heme-containing P450s) was added at the time of IPTG induction. P450 expression was tested at 16 °C, 30 °C and 37 °C after 3 h, 6 h, 12 h, 24 h, 36 h, and 48 h. Ferredoxin expression was not detected.

2.3.3 – Preparation of co-expression constructs

To prepare co-expression constructs, *Xba* I, *Spe* I fragments of the ferredoxin reductase genes from the initial expression constructs (ferredoxin reductase in pET28a) were cloned into the ferredoxin expression vectors at the pET28a *Xba* I site upstream of the cloned ferredoxin to make construct 2. The *Xba* I, *Spe* I fragment includes the

ribosome binding site and His_6 tag of pET28 and so would allow co-expression of both N-terminally tagged genes (the *Spe* I sites were incorporated into initial PCR primers).

2.3.4 - Expression, Purification and Solubilization of StaJ

Expression of StaJ was of the highest yield of all P450s. 1 L cultures of StaJ expressing *E. coli* were harvested by centrifugation, resuspended in 50 mM HEPES pH 7.5, and lysed by passage through the French pressure cell (3 passes at 1000 psi). Lysate was clarified by centrifugation and 1 mL (packed volume) Ni+NTA resin added. Resin and lysate were mixed gently at 4 °C for 1 h before washing 3 times in 50 mM HEPES pH 7.5. Bound protein was eluted by adding the mixture to a 1 mL plastic column and eluting with HEPES pH 7.5, 100 mM imidazole. Fractions collected were 1 mL and expression was detected using 15% SDS-PAGE.

The insoluble fraction after lysis was treated with 1% lauroyl sarcosine for 1 h with mixing at 4 °C. The new insoluble fraction was removed by centrifugation and both soluble and insoluble fractions were examined for StaJ presence by 15% SDS-PAGE. Also the 6 fractions from the Ni+NTA column that were thought to contain StaJ were concentrated 6x (Amicon ultra centrifugal filter device, Millipore).

Solubilized StaJ was subjected to Ni+NTA purification in batch (eluted in 4 mL HEPES pH 7.5, 100 mM imidazole, resin removed by centrifugation) but rapidly became insoluble. White insoluble StaJ present in the eluted fractions was treated with Hemin (8 mg dissolved in 4% triethanolamine added to 2 mg StaJ in HEPES pH 7 at room temperature) and dialyzed overnight against HEPES pH7. Red soluble StaJ was recovered from dialysis and used for subsequent spectrophotometric tests.

2.3.5 - Reduced CO binding difference spectrum of StaJ

StaJ reduction was tested using the sodium dithionite reduced carbon monoxide binding difference spectrum. Two 1 mL plastic cuvettes of StaJ (.25 mg in 1 mL HEPES buffer) were reduced using chips of sodium dithionite (inputs electrons in the absence of reductases), CO was bubbled through one sample (5 mins. at room temperature) and both were scanned over the range of 300 nm to 700 nm using a spectrophotometer and the difference plotted.

2.3.6- 7-ethoxycoumarin biotransformation to 7-hydroxycoumarin by P450s

The co-expression strain (*E. coli* BL21(DE3) carrying pET28 containing SCO1649 and SCO7117) as well as each of the four P450-expressing strains (*E. coli* BL21(DE3) carrying pET28 containing *staF,G,H,J*) were grown at 37 °C to OD600nm 0.6 in 50 ml LB and induced overnight at 30 °C with 1 mM IPTG. Cells were harvested by centrifugation and lysed (in Tris buffer pH 7.5) by 3 passages through a French pressure cell (1000 psi). Ferredoxin-ferredoxin reductase lysate was mixed 1:1 with each P450 lysate and 1 mM 7-ethoxycoumarin added. Each lysate was identically tested alone, as was lysis buffer alone. Samples were incubated at 30 °C (250 rpm) for 36 hours, extracted with 2x volume ethyl-acetate, dried under nitrogen gas then separated by TLC and visualized by fluorescence. Ethyl acetate dissolved 7-ethoxycoumarin and 7-hydroxycoumarin were also analyzed by TLC as standards.

2.4 - Results of P450 Study

2.4.1 - Cloning and Expression of cluster P450s and *S. coelicolor* Proteins

The four P450 enzymes from the A47934 cluster as well as all *S. coelicolor* ferredoxins and ferredoxin reductases were cloned into the expression vector pET28 for over expression in *E. coli*. In addition, the gene encoding a putative flavoprotein in the A47934 cluster (*staR*) was cloned so it could be tested for its ability to act as a ferredoxin reductase or P450 reductase (Table 5). Successful cloning and expression of electron transport enzymes from *S. coelicolor* and P450s from *S. toyocaensis* was the first step toward characterizing the enzyme activity of each P450.

Table 5: Summary of gene cloning and expression tests

Gene	Organism	Product	Cloned into pET28	Mutations	Expression[a]
staF	*S. toyocaensis*	P450	Yes	No	No
Stag	*S. toyocaensis*	P450	Yes	No	Yes
staH	*S. toyocaensis*	P450	Yes	No	Yes
staJ	*S. toyocaensis*	P450	Yes	No	Yes
Sco0681	*S. coelicolor*	Reductase	Yes	No	No
Sco2469	*S. coelicolor*	Reductase	Yes	No	No
Sco7117	*S. coelicolor*	Reductase	Yes	Yes	Yes
Sco0773	*S. coelicolor*	Ferredoxin	Yes	No	No
Sco1649	*S. coelicolor*	Ferredoxin	Yes	No	No
Sco3867	*S. coelicolor*	Ferredoxin	Yes	No	No
Sco5135	*S. coelicolor*	Ferredoxin	Yes	No	No
Sco7110	*S. coelicolor*	Ferredoxin	Yes	No	No
Sco7676	*S. coelicolor*	Ferredoxin	Yes	No	No
Star	*S. toyocaensis*	Flavoprotein	Yes	No	Yes

a: expression was tested under several temperatures (16 °C, 30 °C and 37 °C) and for varying times (3 h, 6 h, 12 h, 24 h, 36 h, and 48 h) No indicates no expression could be detected by Western blot (anti His_6) under any conditions.

Initial expression tests were unsuccessful under many conditions, but the best expression conditions for StaJ (expected size 45 kDa) were found (highest yield). The expressed protein was insoluble (figure 2.5), but was solubilized by treatment with 1% lauroyl sarcosine (figure 2.6). The solubilized His_6-tagged protein was purified by

Ni^{+}NTA chromatography in good yield, but precipitated after detergent was diluted and was white in colour (not indicative of a heme-containing protein).

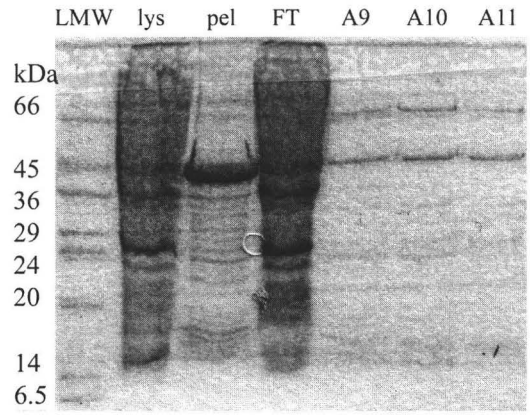


Figure 2.5- Separation of protein by 15% SDS PAGE during StaJ purification. LMW: weight marker, lys: cell lysate, pel: insoluble fraction, FT: unbound to Ni column, A9-11: collected fractions. The predicted StaJ protein is largely insoluble under these conditions.

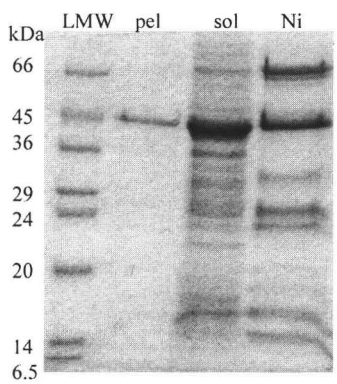


Figure 2.6: Separation of protein by 15% SDS PAGE during solubilization tests of StaJ. Pel: insoluble after 1% lauroyl sarcosine treatment, sol: soluble after treatment, Ni: concentrated A9-11 from figure 2.5. After detergent treatment some StaJ was solubilized.

Hemin was added to the insoluble StaJ (8mg dissolved in 4% triethanolamine added to 2 mg StaJ in HEPES pH 7 at room temperature) and the red colour could not be dialyzed away in HEPES buffer pH 7 (the heme in P450 enzymes is covalently bound to

a conserved Cys residue). The absorbance spectrum of the protein is consistent with a heme containing P450 enzyme (Figure 2.7).

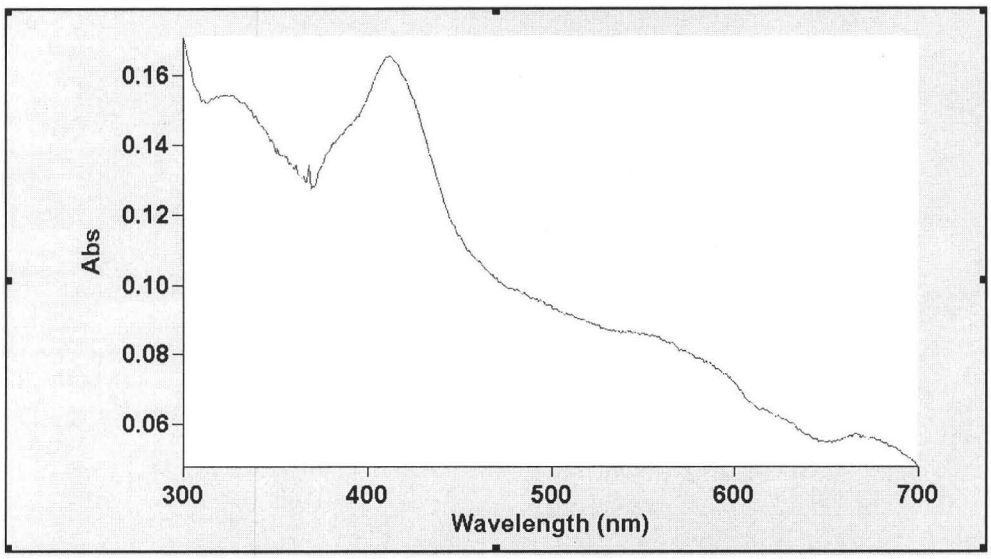


Figure 2.7- Absorbance spectrum of purified StaJ after hemin treatment showing characteristic heme-protein peak at 419 nm.

When reduced chemically with sodium dithionite (few chips in cuvette) and bound to CO, an absorbance peak near 450 nm (455 nm) was observed (a property of P450 monooxygenases that gave rise to their naming, Figure 2.8) (51, 52). This result gave confidence that the purified StaJ could accept electrons and thus may be active. In light of the fact the substrate was unknown, this is the only indication of proper protein folding and function we could base this decision upon.

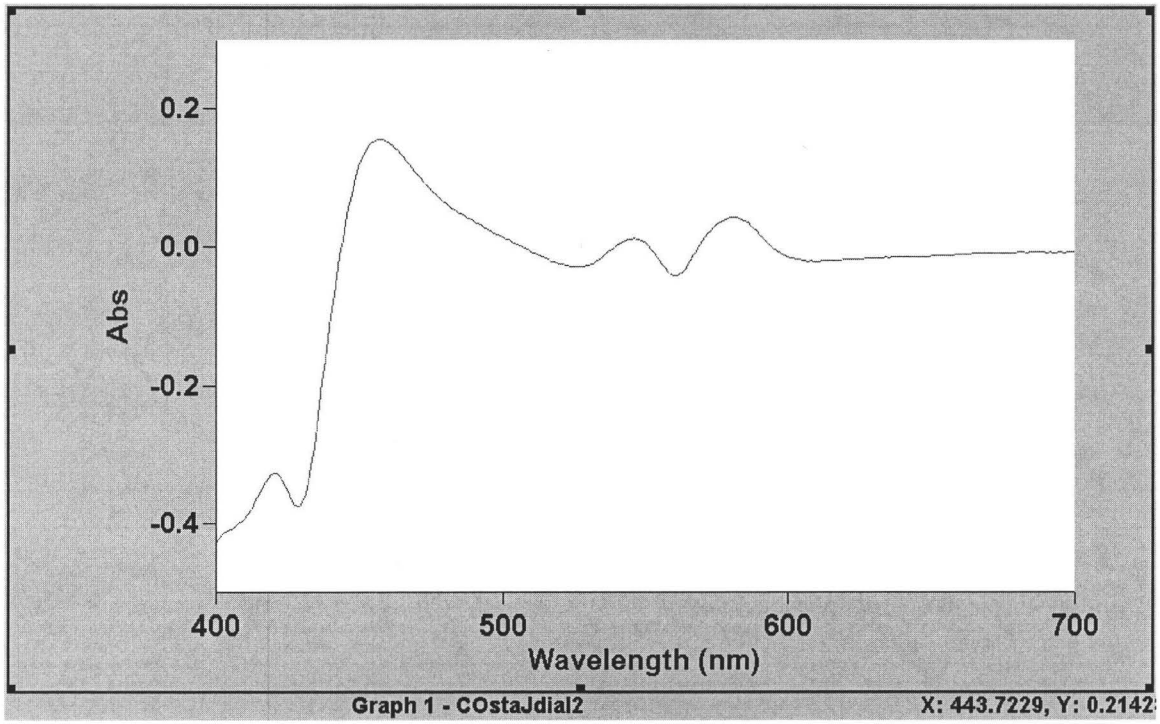


Figure 2.8- Reduced CO binding difference spectrum of StaJ showing a characteristic peak at 455 nm. Two cuvettes containing StaJ were reduced with sodium dithionite and CO bubbled through one sample and the difference spectrum recorded.

7-ethoxycoumarin biotransformation to 7-hydroxycoumarin by P450s

The ferredoxin and ferredoxin reductase co-expression constructs were tested for their ability to donate electrons to the P450s. It should be noted that without a positive *S. griseolus* P450 control (not successfully cloned) it is unknown whether the reactions failed to metabolize 7-ethoxycoumarin due to substrate specificity issues of insufficient electron transport. The TLC results are shown in figure 2.9

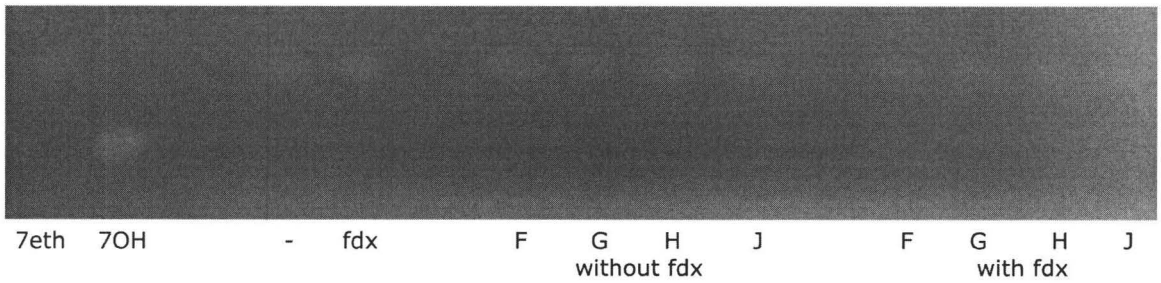


Figure 2.9- TLC analysis of ethyl acetate extracts of StaF,G,H,J lysates with and without added ferredoxin and ferreoxin co-expression lysate after 36 h incubation with 7-ethoxycoumarin. 7eth: 7-ethoxycoumarin, 7OH: 7-hydroxycoumarin, fdx: ferredoxin and ferredoxin reductase lysate, F,G,H,J: StaF,G,H,J lysates respectively.

The lack of biotransformation may be attributed to insufficient electron transport or due to the cluster P450s inability to catalyze this reaction or due to poor expression. StaF, StaG and SCO1649 seem to have expressed but the other enzymes may not have as seen by SDS-PAGE in figure 2.10.

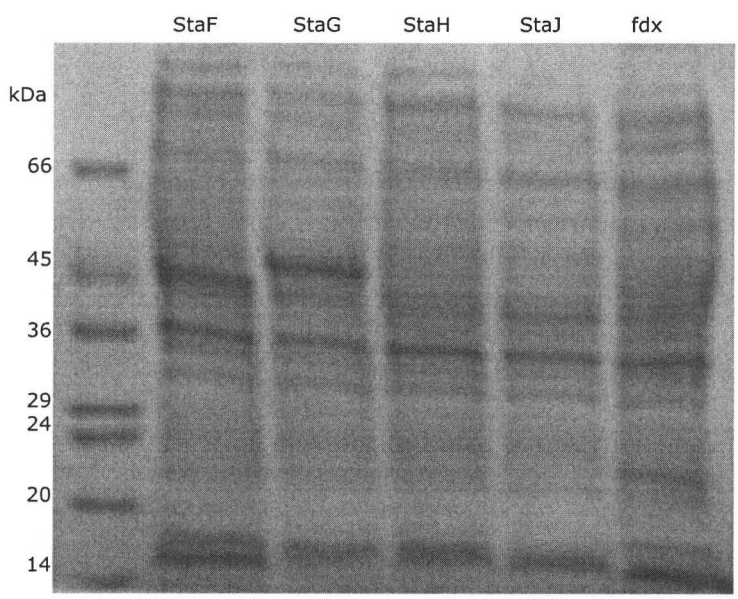


Figure 2.10- 11% SDS-PAGE separation of cell lysates after IPTG induction overnight at 30 °C. fdx: SCO1649-SCO7117 co-expression construct.

2.5- P450 Project Conclusions and Future Work

Coincident with the time that StaJ was successfully purified and shown to be active using the sodium dithionite reduced CO binding difference spectrum, OxyB *in vitro* activity was reported in the literature (79). Because the impact of replicating the experiment would be low and the monetary and man hour costs high, it was decided to halt work on the cross-linking P450 project. StaJ is homologous to OxyC, the last P450 predicted to act in the cross-linking pathway and the other P450s were not expressing in high yield.

The failure of the cluster P450s to metabolize 7-ethoxycoumarin may be attributed to a number of reasons. The enzymes could be incapable of catalyzing this reaction due to specificity of the active site. The electron transport enzyme from *S. coelicolor* could be insufficient to drive catalysis, the P450s could also be expressed in an inactive form. In addition, SDS-PAGE analysis indicates poor or lack of expression of SCO7117 (ferredoxin reductase) which would make the whole system inactive.

There is still much work to be done in this area and the project may be revisited in terms of subsequent cross-linking and the ordering of the StaG-mediated cross-link. The successful construction of the co-expression vectors provides the reagents necessary for future studies.

Section 3- Investigating methods of inhibiting the GPA resistance mechanism

The second Study in this thesis

3.0 - Introduction to Inhibiting the GPA Resistance Mechanism

Although inhibitors of VanHAX activity would be useful for combination therapy, none have come to clinical use. There is a wealth of information about two component regulatory systems and the kinase activities associated with them, but very little is known about what actually triggers VanS autophosphorylation in the vancomycin resistance systems. The VanA and VanB resistance phenotypes differ in their ability to confer resistance to teicoplanin, but confer the molecular mechanism, substitution of D-Ala-D-Lac for D-Ala-D-Ala, is identical. Since the mechanism of resistance is the same in both phenotypes, it is conceivable that differential resistance to teicoplanin and vancomycin is a result of the structural differences between the classes of GPAs, and ultimately, their ability to initiate VanS autophosphorylation. Because of this specificity, we predicted that there may be a protein that has the function of recognizing the subtle variation between GPAs. It is reasonable to expect that VanS itself performs this function as there is a variable domain in the sequenced *vanS* genes that is predicted to encode an extracellular domain that could act as a GPA recognition site. Although some hybrid *vanS* genes have been inserted into the genome of various strains, still very little is understood about GPA specificity. It has also been shown that some GPAs bind (or inhibit) penicillin-binding proteins (PBPs) such as PBP2 in *S. aureus*, which has also been shown to be essential for vancomycin resistance in that strain (63). It is plausible that multiple proteins and perhaps even lipid intermediates that accumulate due to GPA inhibition of peptidoglycan biosynthesis are involved in GPA recognition.

Chromatographic studies using hydrophobic vancomycin derivatives (similar to oritavancin) bound to the solid phase showed that virtually all PBPs and some other peptidoglycan modifying proteins from *E. coli* were retained specifically on the columns, but were not retained on regular vancomycin columns (64). In addition, the hydrophobic biphenyl constituents were shown to retain a similar combination of proteins in the absence of GPA when bound to the same resin, indicating that the chlorobiphenyl group (similar to benzophenone to be discussed further) may play a major role in PBP binding or inhibition (64).

3.1-Goals of GPA-binding project

In light of the lack of information about the molecular events leading to VanS autophosphorylation, our goal was to first identify GPA-binding proteins and elucidate how each affects GPA-resistance. Ultimately, if we could find a compound that could bind to these proteins, but not activate GPA resistance, we would have a promising lead for combination therapy.

To find GPA binding proteins, we decided to prepare GPA derivatives harbouring a photolabile benzophenone moiety that, upon photolysis, would result in covalent cross-linking between GPAs and binding proteins. These derivatives were also synthesized with fluorescent tags for visualization or biotin for streptavidin-agarose purification and detection via Western blot with anti-biotin antibodies.

The benzophenone group is photolabile and when irradiated with light at ~355 nm, electron rearrangement allows the group to insert itself into CH bond with a range ~4-5 Å (Figure 3.1).

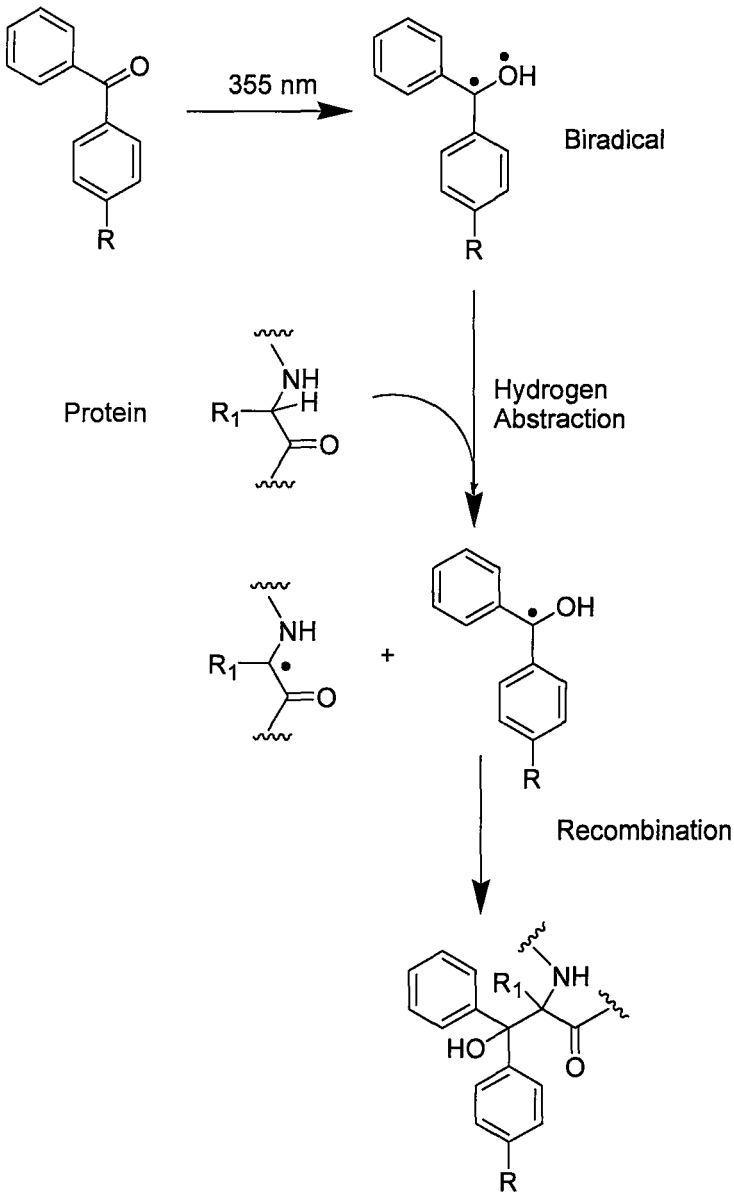


Figure 3.1- Mechanism by which benzophenone inserts into CH bonds. Benzophenone was used to covalently bond our GPA derivatives to any proteins that bind them. Adapted from reference (1).

Shown below are the structures of the GPA derivatives used in this study. These include benzophenone-vancomycin-lysine-biotin (Bh-vanc-Lys-Btn), benzophenone-vancomycin-lysine-biotin-BODIPY (Bh-vanc-Lys-Btn-Bod) and benzophenone-A47934-

Biotin (Bh-A47934-Btn). All of these compounds were synthesized by Dr. Kalinka Koteva and the structures are shown in figure 3.2.

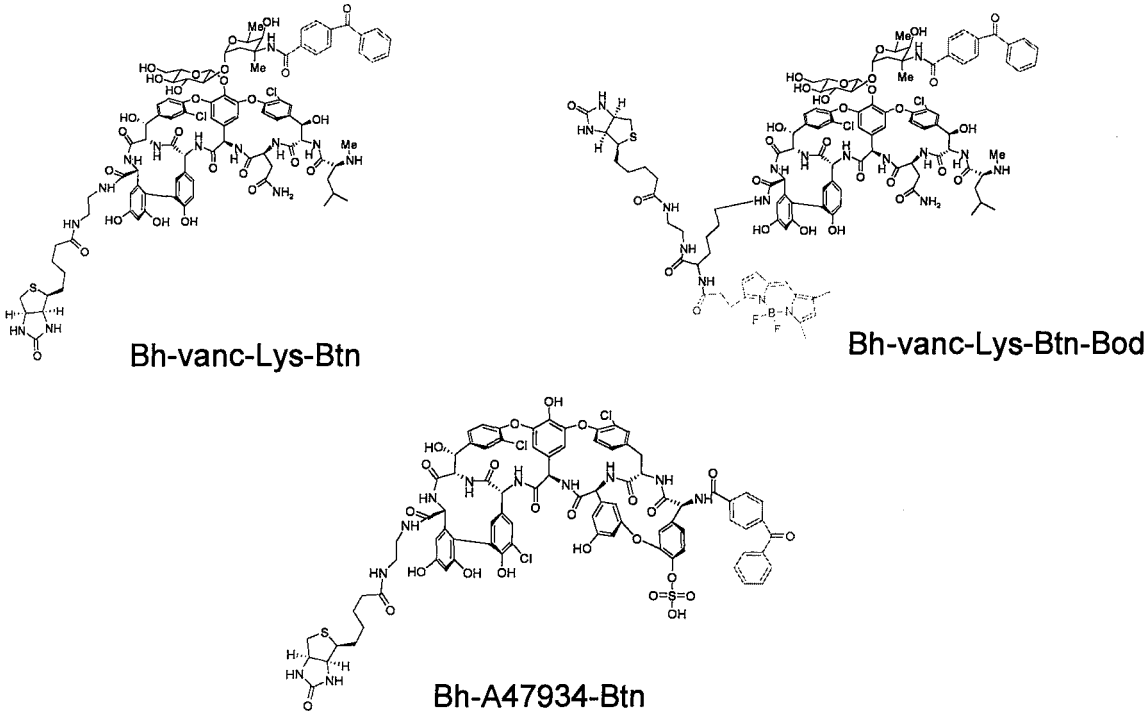


Figure 3.2- Structures of GPA derivatives used in this study. Bh-Benzophenone, vanc-vancomycin, Btn-Biotin, Bod-BODIPY, Lys-lysine linker.

By incubating and photolyzing these compounds with membrane fractions from strains that are resistant to the derivatized antibiotic and those that are not, we hoped to identify proteins that are directly involved in resistance.

Proof of principle for this approach comes from work with radiolabelled penicillins used to find PBPs in the 1970s. Penicillins become covalently linked to PBPs as part of their inhibition mechanism and so do not require a photolabile group to be added. Although our approach modifies the structure of the antibiotic, the benzophenone

group was added at an analogous position on vancomycin as the chlorobiphenyl group in Oritavancin, in hopes of minimizing the effects of derivatization. The compounds used also retain some antimicrobial activity, adding to our confidence that their behaviour would be similar to the underivatized compounds.

Photoaffinity labeling of biomolecules has been in practice since the 1970's (22, 23). There are numerous photolabile groups to choose from differing in photosensitivity and what bonds into which they can insert (6). We chose to use the benzophenone moiety because of its preferential insertion into CH bonds that would be useful for protein targeting and for its stability under ambient light conditions (25).

3.2-Materials and Methods

3.2.1 – Bacterial strains, growth conditions and membrane preparation

S. coelicolor and *S. toyocaensis* NRRL 15001 were grown in 4 L baffled flasks containing 1 L tryptone soya broth medium (TSB, Oxoid) for 3-4 days at 30 °C with shaking at 250 rpm. *B. subtilis* and *E. coli* were grown in 4 L flasks containing 1 L LB medium overnight at 37 °C with shaking at 250 rpm. 1 L cultures were harvested by centrifugation, resuspended in 50 mM phosphate buffer pH 7 and lysed by three passages through a French Pressure cell (1000 psi). Cellular debris was removed by centrifugation and clarified lysate was kept at 4 °C. Membrane fractions were collected by ultracentrifugation of the clarified lysate (100,000 g for 35 mins. at 4 °C). Membranes were washed 3 times in 50 mM phosphate buffer pH 7 by pelleting by ultracentrifugation after each wash (100,000 g for 35 mins. at 4°C). Fractions were resuspended by pipetting and diluted to a protein concentration of 5 mg/ml in 50 mM phosphate buffer pH 7. Membrane fractions were stored in 10% glycerol at -20 °C. Protein concentrations were determined by the method of Bradford (13).

3.2.2 - Method 1: Using BODIPY-7-aminocephalosporanic acid to label PBPs

800 µl of membrane fraction (*E. coli* and *S. coelicolor*) were incubated with 2 µg (3.6 nmol) of 7-aminocephalosporanic acid-BODIPY (7-aca-BODIPY) at 30 °C for 15 and 30 minutes. Proteins were separated by 11% SDS PAGE and visualized by in-gel fluorescence scanning (Typhoon), exciting at 505 nm and detecting emission at 513 nm (the excitation and emission maxima for BODIPY FL used).

3.2.3 - Method 1a: Using Bh-vanc-Lys-Btn-Bod to detect vancomycin binding proteins

Bh-vanc-Lys-Btn-BODIPY was incubated with membrane preparations of *S. coelicolor* and *S. toyocaensis* for 15 mins. at 30 °C, and then irradiated with high intensity light at ~355 nm for 30 seconds at room temperature (200 W Hg/Xe lamp through two Corning 7-51 filters) in 1 mL plastic cuvettes (1 cm path length). The irradiated samples were separated by 11% SDS PAGE and visualized by in-gel fluorescence scanning as in method 1.

3.2.4 - Method 2: Enriching post photolysis samples for biotinylated products

In light of poor signals from methods 1 and 1a, it was decided to enrich GPA binding proteins via their biotin tags using streptavidin-agarose beads (EZview red streptavidin affinity gel, Sigma) and performing Western blots using anti-biotin antibody to amplify the weak signal. After photolysis, samples were incubated with 10 µl streptavidin-agarose beads for 1 hr. at 4 °C with occasional shaking before washing 3 times with 750 µl 50 mM phosphate buffer pH 7. Bound proteins were eluted by boiling in SDS PAGE loading buffer (10% SDS, 20% glycerol, 0.005% Bromophenol Blue, dithiothreitol in Tris buffer pH 6.8) and separated by 11% SDS PAGE before electrophoretic lateral transfer to polyvinylidene fluoride (PVDF) membranes. Western blots were blocked overnight in blocking buffer (Tris-buffered saline pH 7.5, 1% non-fat milk at 4 °C), incubated at room temperature 1 hr in blocking buffer containing 4 µl anti-biotin antibody (derived from goat, Sigma), washed 4 times (15 mins. each) in Tris-

buffered saline, 1% Tween-20 (TTBS, pH 7.5), then incubated 45 mins. in blocking buffer containing 2 µl rabbit anti-goat IgG (horseradish peroxidase conjugate, Sigma) and washed 4 more times in TTBS. Western blots were developed by immersion in Western Lighting (Perkin Elmer) mix containing luminol and hydrogen peroxide. BioMax films (Kodak) were exposed for 2 seconds and developed in an automated film developer.

3.2.5 - Method 3: Depletion of background signal with streptavidin-agarose and Using Stringent Washes before elution of biotinylated proteins from resin

Due to suspected non-specific binding to the streptavidin-agarose, streptavidin-agarose depletion and more stringent washes were used. The protocol remains the same as method 2 except the washes prior to elution from the resin were with citrate buffer pH 4, and borate buffer pH 8 before phosphate buffer pH 7, 500 mM NaCl. In addition the membrane samples were pre-incubated with 50 µl of streptavidin-agarose 4 times for 1 hr at 4 °C (occasional shaking) prior to experiments.

3.3-Results of GPA binding experiments

Initially the goal was to use in-gel fluorescence scanning to visualize GPA binding proteins. In order to verify our system, we tested BODIPY-7-aca using *E. coli* and *S. coelicolor* membrane fractions to ensure PBPs could be detected. The structure of BODIPY-7-aca is shown below in figure 3.3.

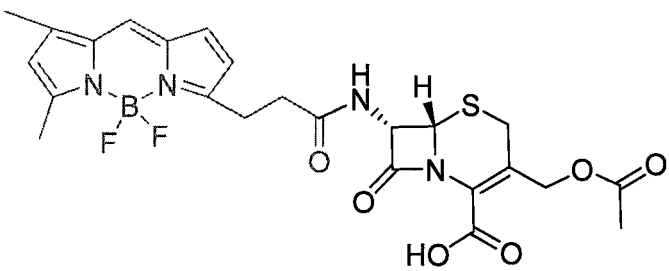


Figure 3.3- Structure of BODIPY-derivatized 7-aminocephalosporanic acid.

Figure 3.4 shows the in gel fluorescence scan of each membrane preparation with 15 min. and 30 min. incubations before gel loading as per method 1. We are able to detect high molecular weight PBPs in *E. coli* and *S. coelicolor* membrane preparations.

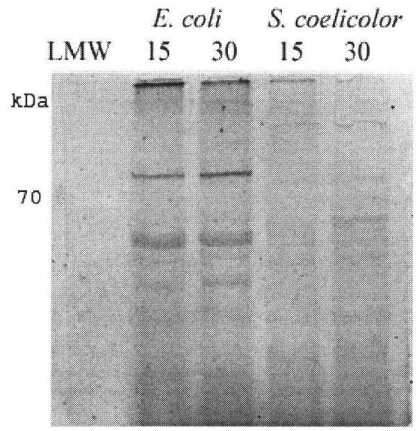


Figure 3.4- 7aca-BODIPY binding experiment. Separation of membrane fraction proteins by SDS PAGE, PBPs visualized in gel by Typhoon scanner (excite 505nm, emit 513nm). Ld: ladder (not visible except for 70 kDa band), 15: 15 min incubation, 30: 30 min incubation, E. coli: *E. coli* membrane fractions, S. coel: *S. coelicolor* membrane fractions

Because the PBPs were visible, the experiment was repeated using Bh-vanco-lys-Btn-BODIPY and membrane fractions from *S. toyocaensis* and *S. coelicolor* with photolysis as per method 1a. Visible bands in the test lanes are similar in size to PBPs as seen in figure 3.5 below.

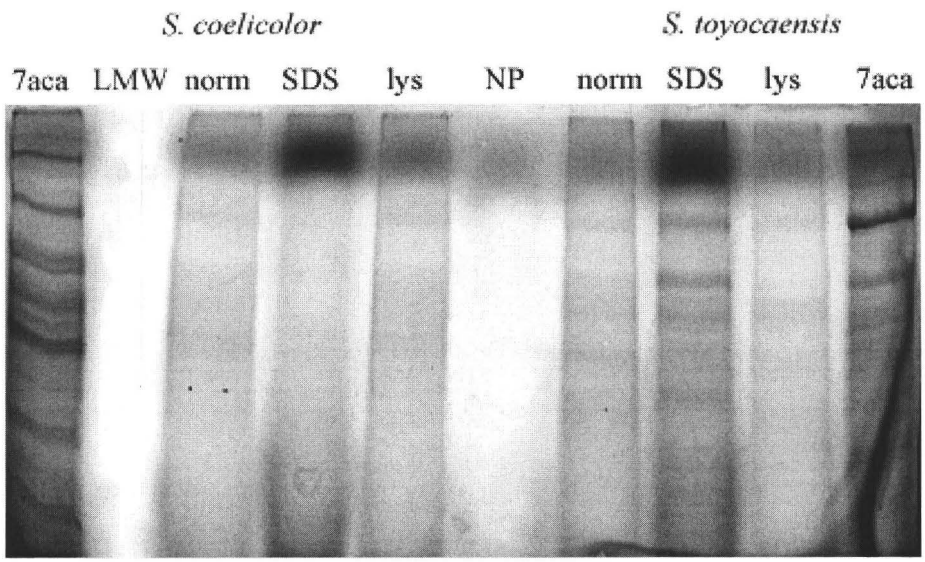


Figure 3.5- Bh-vanco-BODIPY binding experiment. 800 µL of *S. coelicolor* and *S. toyocaensis* membrane fractions were treated with 2 µg Bh-vanco-BODIPY, separated by 10% SDS PAGE and visualized by in gel fluorescence scanning after photolysis in the presence of benzophenone-BODIPY conjugated vancomycin. 7aca: 7-aca treated membrane fractions to detect PBPs for size comparison. LMW: marker (not visible in fluorescence scan); Norm: membrane fractions treated with compound and photolyzed; SDS: membrane fraction pre-treated with SDS to denature proteins; lys: lysozyme treated membrane fraction (will hydrolyze peptidoglycan).

This result was very promising. We could detect proteins that seem to bind Bh-vanco-lys-btn-BODIPY and some are of similar size to the bands seen in the 7-aca lanes. The fact that PBPs seem to be visible in this experiment is not surprising given the fact that it has been shown that GPAs inhibit transglycosylase activity, hydrophobic vancomycins (chlorobiphenyl, similar to benzophenone group) bind PBPs and the PBP2 is implicated in GPA resistance in *S. aureus* (20, 63, 64).

The bands were quite weak in intensity so it was decided to use streptavidin-agarose resin to enrich for proteins that have covalently cross-linked GPA derivatives and to use anti-biotin antibody to specifically amplify the signals by Western blot as described in method 2. The anti-biotin antibody was tested with Biotin-7-aca to ensure

the biotinylated compounds could be used to detect PBPs as the fluorescence system. A biotinylated protein ladder was also used as a positive control for Western analyses; these results are shown in figure 3.6.

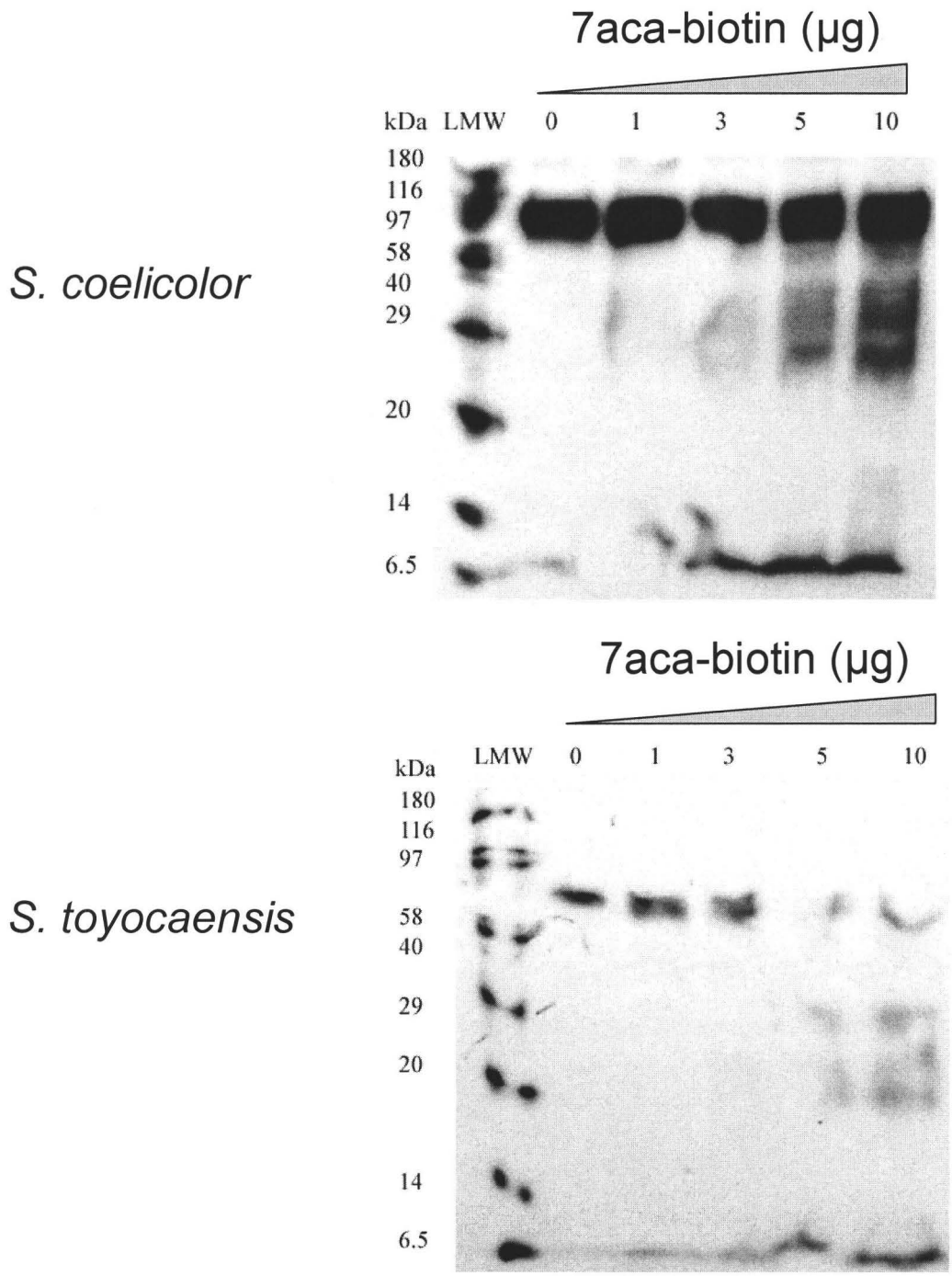


Figure 3.6- 7aca-biotin binding experiment. 11% SDS-PAGE separation of *S. coelicolor* and *S. toyocaensis* membrane fractions after treatment with various concentrations of biotin-7-aca. Visualization by Western blots using anti-biotin antibody. 0-10: increasing amounts (in µg) of biotin-7-aca.

The presence of a band in the 0 μg lane was puzzling, but the Western analyses gave a much more intense banding pattern for PBPs (compared to figure 3.4, especially for *S. coelicolor*). Since the PBPs seemed to be visible at higher concentrations of 7-aca-BODIPY, we opted to continue with the biotin detection system. The biotinylated compounds were used to detect vancomycin binding proteins using the GPA derivatives via Western analysis as illustrated in figure 3.7 below.

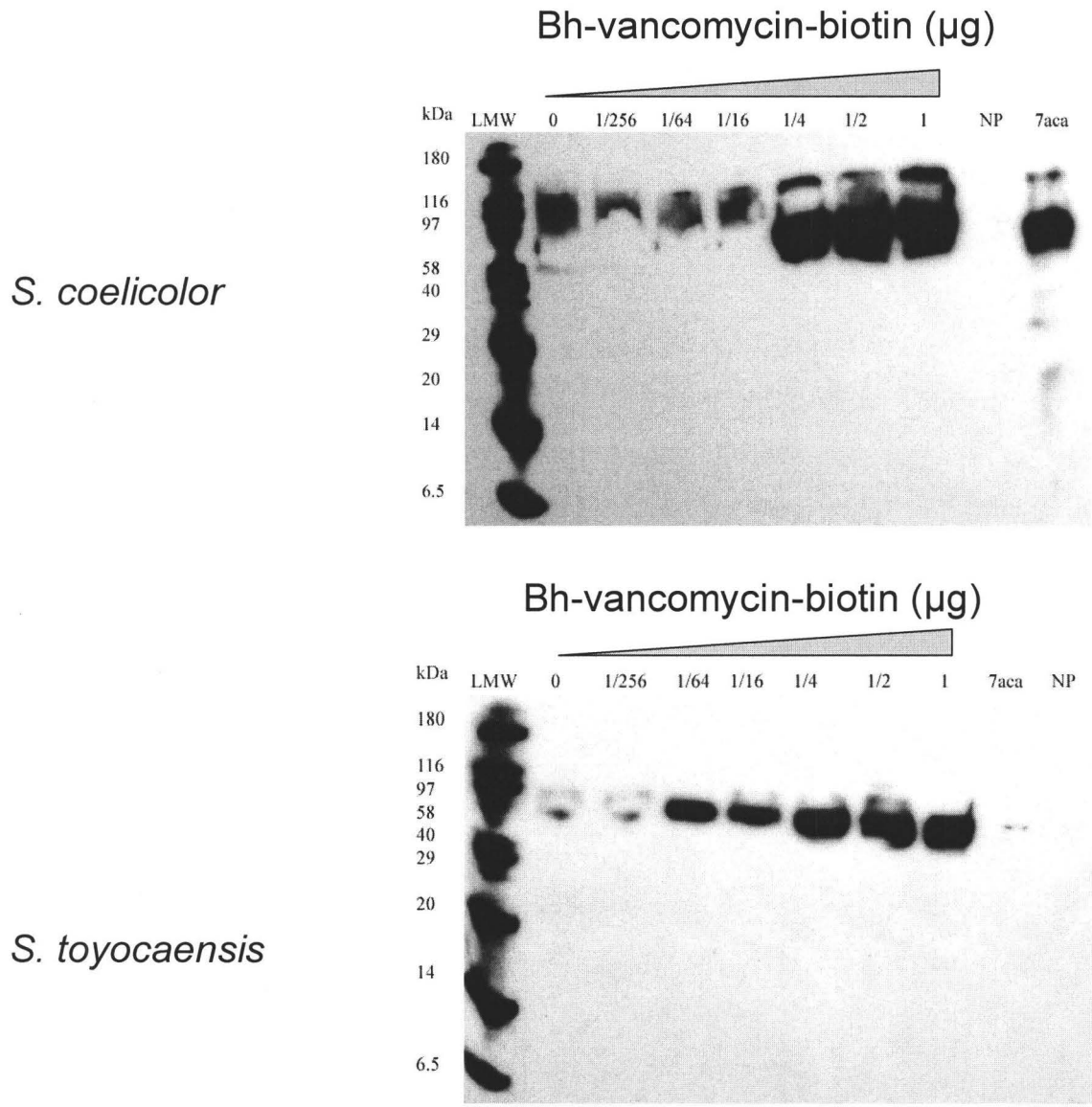


Figure 3.7- Bh-vanco-biotin binding experiment. Western blots of *S. coelicolor* and *S. toyocaensis* membrane fractions treated with increasing amounts (0-1 µg) of Bh-vanc-Biotin. 7-aca: treated with 7-aca-biotin, NP: no membrane fraction, water and compound only.

These results looked promising as a dose-dependent signal was visible, although signal was still present in the 0 µg lanes and the PBPs were not as visible in the 7-aca-biotin lanes. Competition experiments were performed to determine if the signal could

be out competed by unlabelled vancomycin. Very high concentrations of vancomycin were necessary to eliminate bands and may have interfered with the experimental system. The results of the competition experiment are shown in figure 3.8.

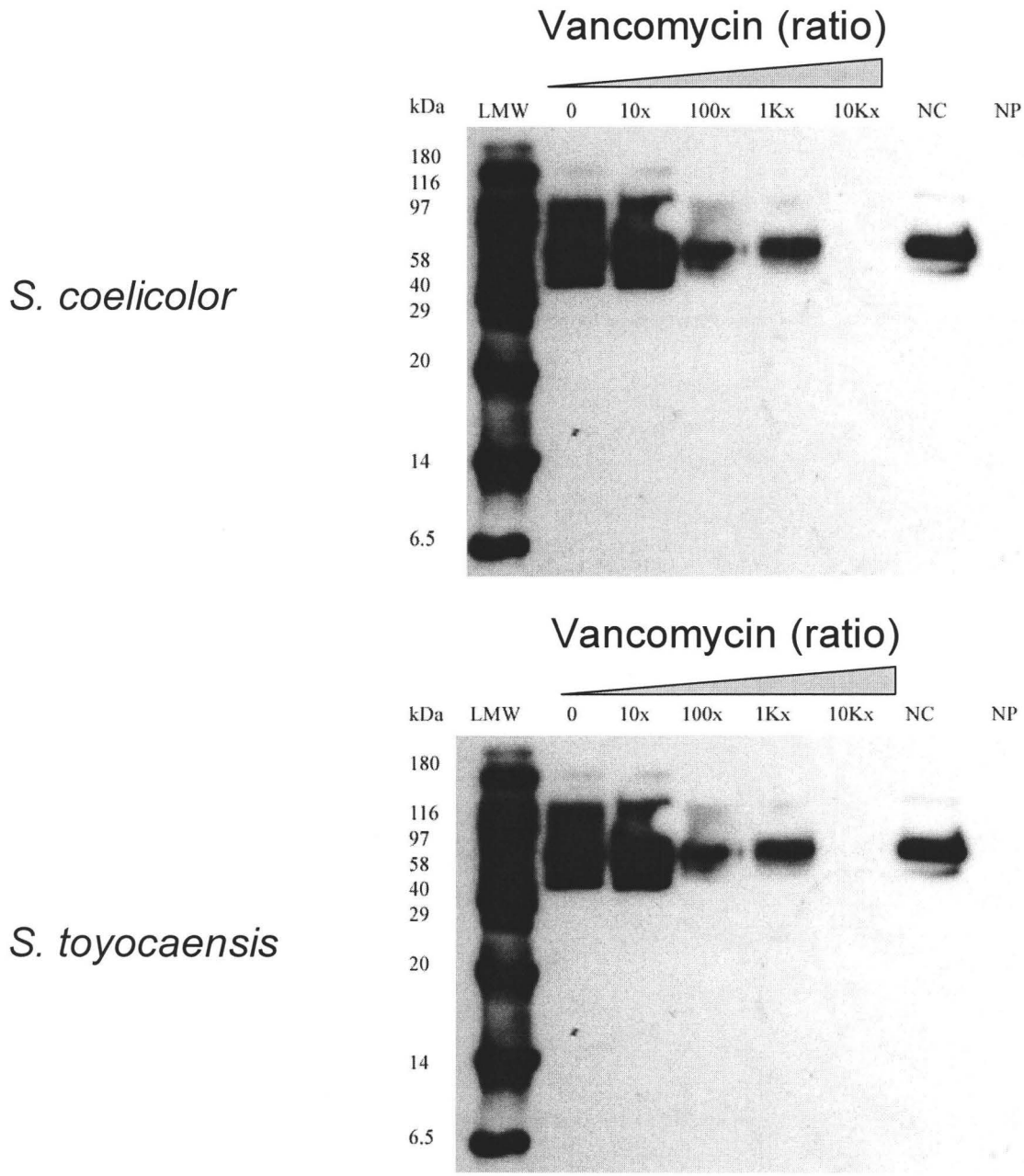


Figure 3.8- Bh-vancomycin-biotin competition experiment. Competition experiment where membrane fractions were treated with 1μg of Bh-vanc-biotin and increasing concentrations of unlabelled vancomycin (10x = 10μg etc.) NC: no Bh-vanc-biotin added, only membrane fraction, NP: no membranes added, only Bh-vanc-biotin.

The presence of a signal with no biotinylated compound was unexpected but because the experiments met our expectation that vancomycin could compete for the

signal. We decided to repeat the experiments using Bh-A47934-biotin. The results were similar to those for vancomycin derivatives with an apparent dose-dependent signal and persistent signal in the absence of biotinylated compounds. Missing signal in ¼ lane and light signal in 1/2 lane is attributed to loading error. The results are shown in figure 3.9.

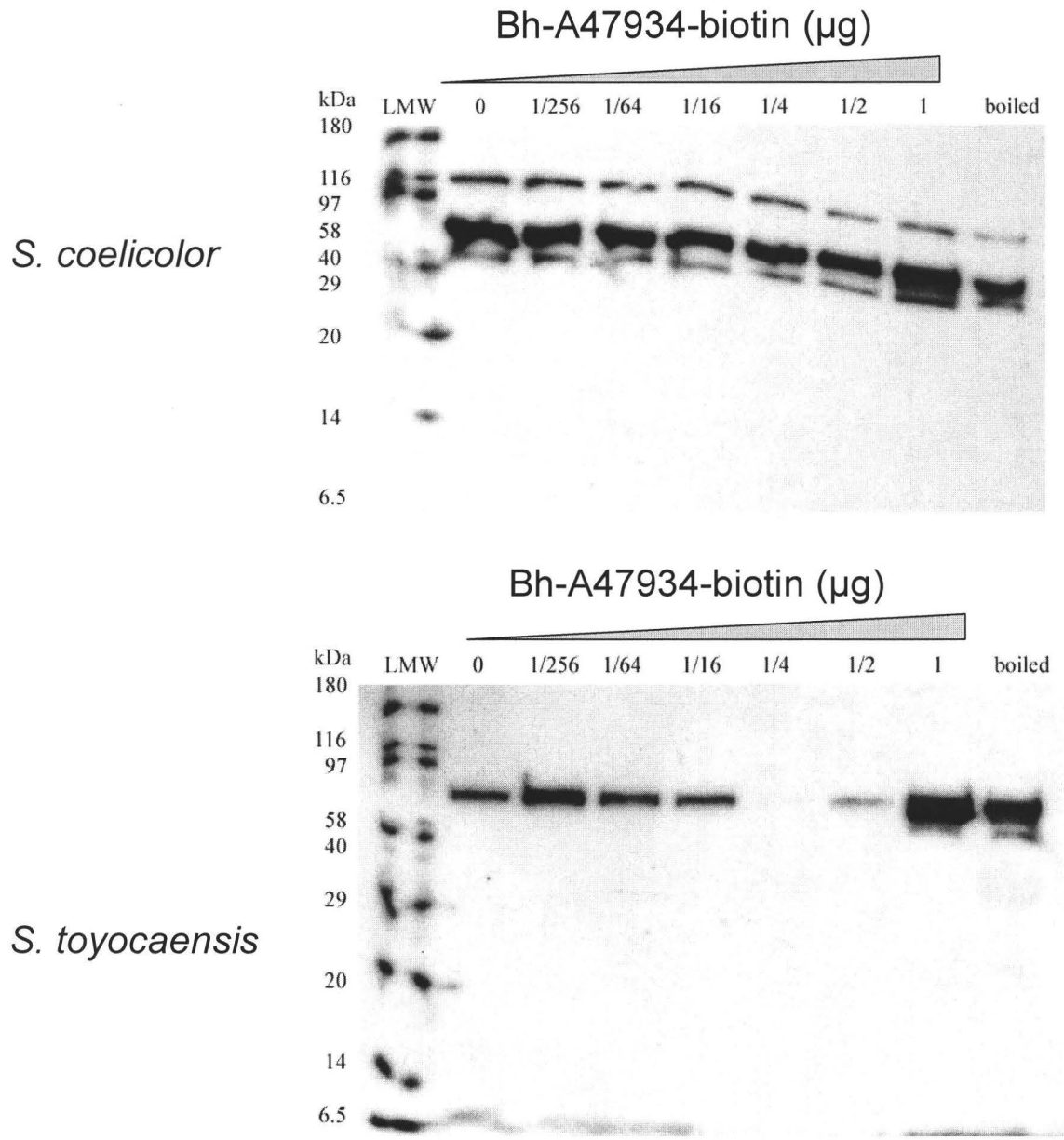


Figure 3.9- Bh-A47934 binding experiment. Western blots using anti-biotin antibody against membrane fractions treated with increasing concentrations (in µg) of Bh-A47934-biotin. Boiled: membrane fractions were boiled in SDS PAGE loading buffer prior to incubation with test compound. Moderate dose dependence is apparent, again, signal persists in absence of biotinylated compound, and even when membranes were boiled in SDS.

In light of the fact that signal remained in the absence of biotinylated test compounds, it was decided to attempt binding experiments with *B. subtilis*, which we did

not expect would express any vancomycin resistance genes, but would still likely express PBPs capable of binding our GPA derivatives. Results for the binding of Bh-vanc-Btn are shown in figure 3.10.

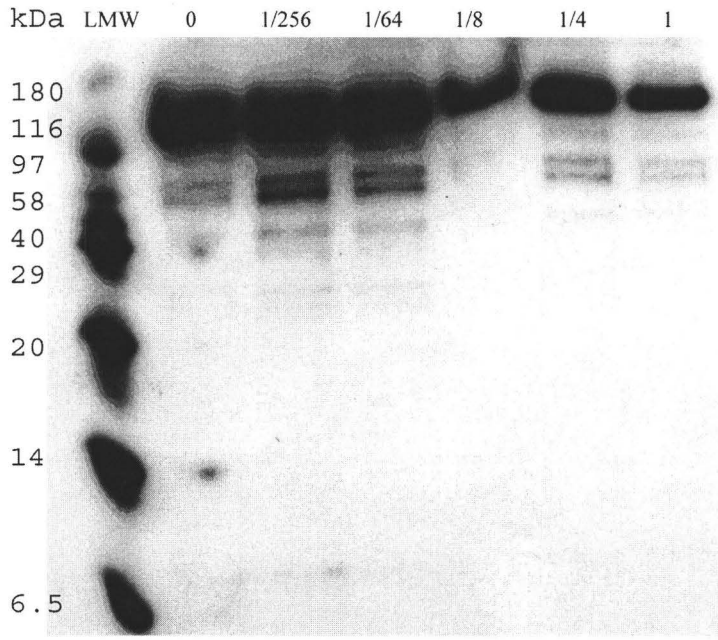


Figure 3.10- Bh-vanco-biotin binding experiment with *B. subtilis*. Western blot of *B. subtilis* membrane fraction treated with increasing Bh-vanc-Lys-Biotin concentrations (0-1 μg as shown at top).

The persistent presence of signal in the absence of biotinylated compound was vexing. We decided to perform Western blots on membrane fractions that had never been in contact with the biotinylated compounds to test the theory that minuscule amounts of contamination was sufficient to give signal. Dilutions of the membrane preparations were made to show that in fact something in the samples was the cause for signal, the results are illustrated in figure 3.11. The lack of signal in the 1/100 lane is attributed to loading error.

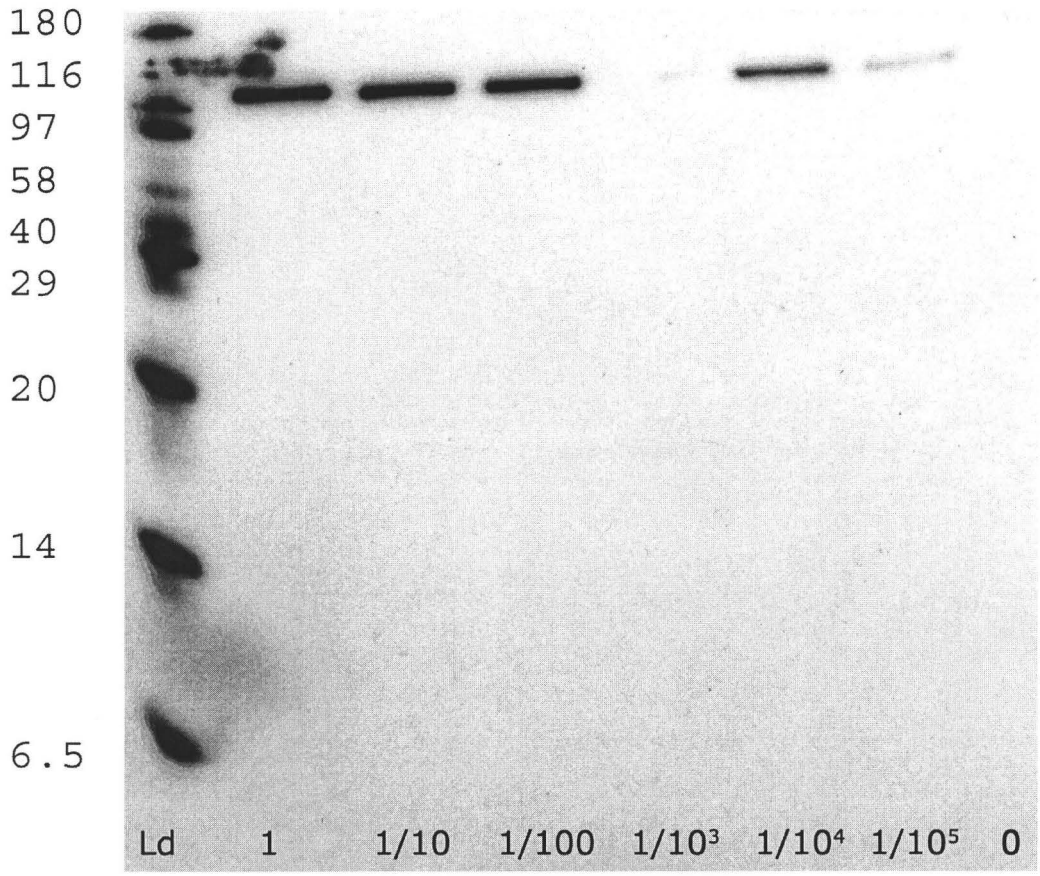


Figure 3.11- Western blots using anti-biotin antibody on various dilutions of *B. subtilis* membrane preparations that were not treated with any GPA or derivative. 1= 1 μg/μl, others are dilutions as indicated.

In an attempt to eliminate the apparent background signal, streptavidin-agarose was used to deplete the membrane samples. The signal remained in the lanes lacking compound in both *B. subtilis* and *S. coelicolor* as shown in figure 3.12.

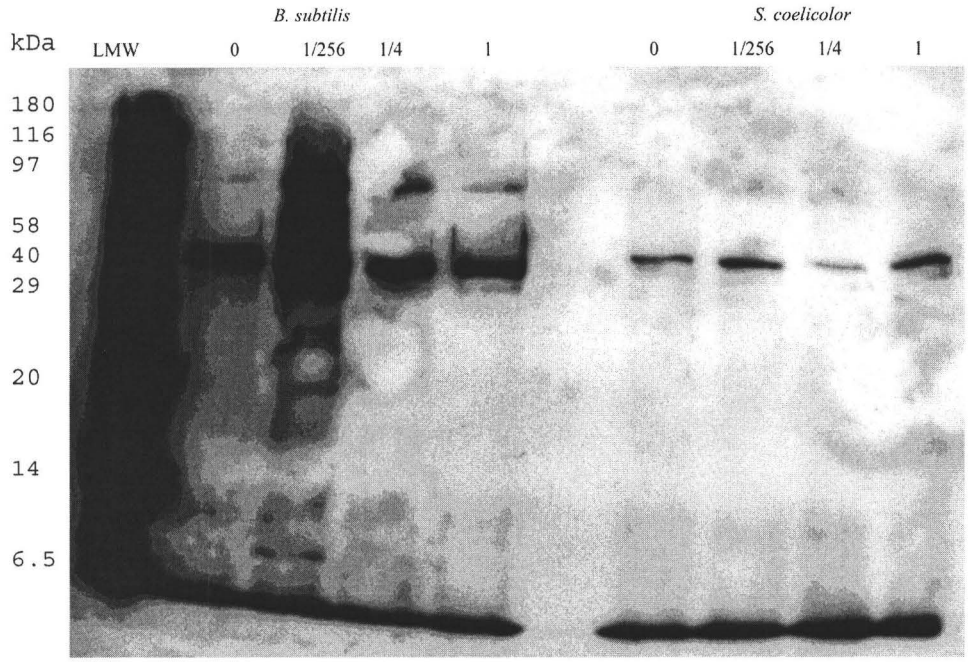


Figure 3.12- Pre-depleted Bh-vanco-biotin binding experiment. *B. subtilis* and *S. coelicolor* pre-treated with 50 µL streptavidin-agarose 3 times before the membrane fractions were treated with Bh-vanc-Lys-Biotin (in µg as indicated at top). Biotinylated proteins visualized by Western blot using anti-biotin antibody.

It was at this time decided to attempt to deplete the membrane fractions repeatedly using streptavidin-agarose resin (4 rounds of depletion using 5-times as much streptavidin-agarose as used to enrich for biotinylated proteins) and in addition to use a stringent wash system to eliminate background signal as in Method 3. Although the background was greatly reduced, it was still not eliminated as evident in figure 3.13.

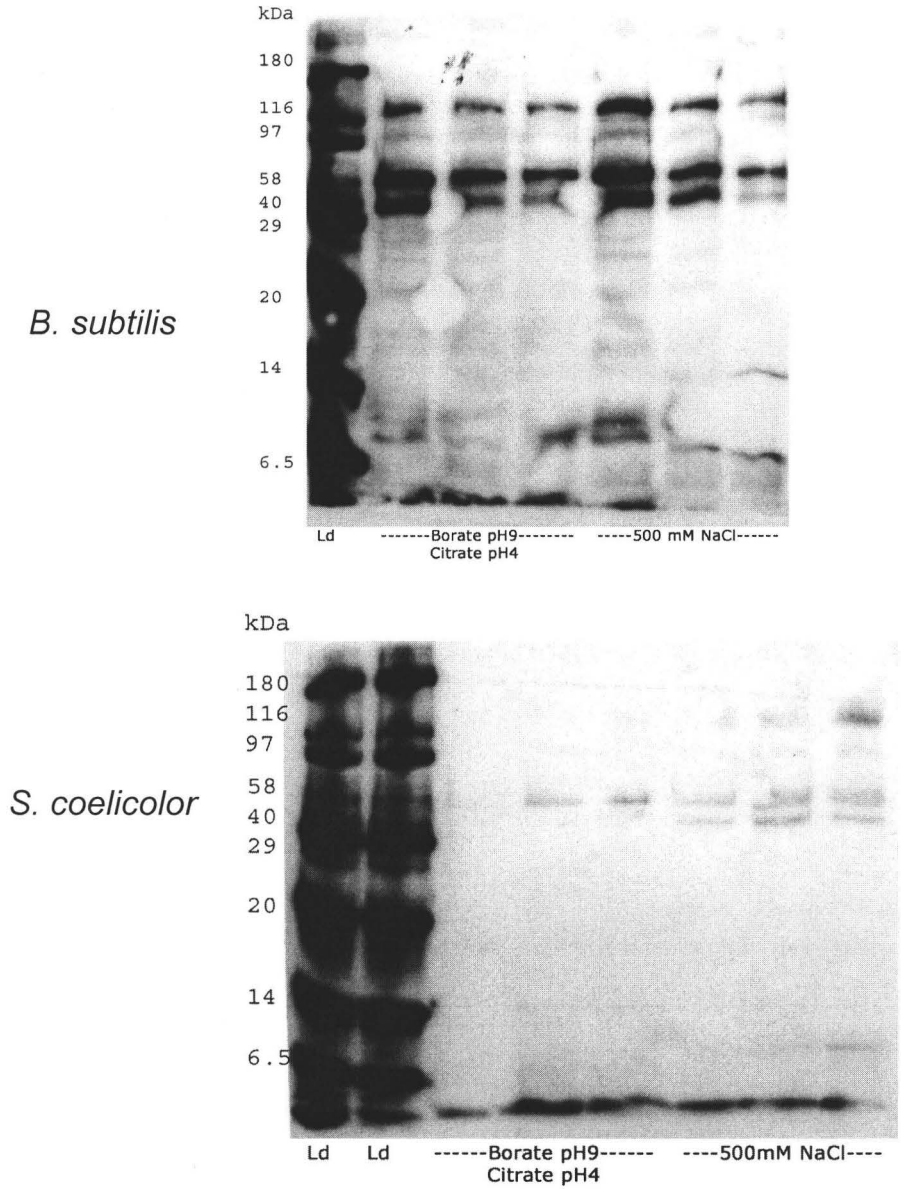


Figure 3.13- *B. subtilis* and *S. coelicolor* membrane fractions pre-treated with streptavidin-agarose resin to deplete background signal, then subjected to stringent washing before elution from streptavidin beads, these samples were not treated with Bh-vanc-Lys-Biotin. All lanes of a single treatment have the same amount of sample loaded showing variability between lanes.

Our inability to remove the background signal and the fact that the detection limit for anti-biotin antibodies is 10^{-14} moles of protein, we concluded the study at this step as any specific bands should have been seen.

3.4-Conclusions and Future Work

Because we could not obtain specific bands in any of our photolysis experiments, it must be our conclusion that under our conditions, there are no proteins that bind to the GPA derivatives and become cross-linked upon photolysis. This is not to conclude that GPA binding proteins do not exist, but rather that our system was insufficient to detect them. This could be due to several factors.

The modifications made to the GPAs to make them amenable for our purposes may have interfered with the binding to membrane proteins responsible for detecting the GPAs. This could be tested by several means including RT PCR or Northern blots to detect mRNAs for the *vanHAX* genes in response to GPA presence. Another approach could be to put a reporter gene such as firefly luciferase under control of the *vanH* promoter (the promoter that phosphorylated VanR acts upon to stimulate the resistance response). This could tell us that our GPA derivatives are sufficient to induce the resistance genes and thus must also be recognized by any GPA binding protein involved in resistance.

Another possibility is that the preincubation with the GPA derivatives was inadequate to allow for proper docking of the compound to the receptor protein. Because the initial experiments with in gel fluorescence scanning looked promising, the incubation of 15 minutes was used throughout the study. If the kinetics of the binding and activation of *vanHAX* were known, we could be more confident in our preincubation times.

The possibility that there is no GPA recognition protein does exist, it has been repeatedly suggested in the literature that it is an accumulation of peptidoglycan lipid intermediates that triggers the autophosphorylation of VanS. We expected to detect PBPs in our experiments as the benzophenone group is on the sugar residue suspected to interact with and inhibit the transglycosylation function of PBPs, but we did not. This anomaly leads to the suspicion that it is an inherent flaw with our system that led to lack of results.

S. coelicolor expresses 3 biotinylated proteins (14, 43). The largest (145 kDa) is as of yet uncharacterized but is proposed to be involved in fatty acid biosynthesis as is the 88 kDa propionyl-CoA carboxylase α-subunit also found in *S. coelicolor*. The final biotinyated protein expressd by *S. coelicolor* is 70 kDa and has been speculated to be the α-subunit of an inactive Acetyl-CoA carboxylase of pyruvate carboxylase (14). It is likely that similar proteins are founding *S. toyocaensis*, but it is somewhat surprising to observe such large biotinylated proteins in *B. subtilis* when the only biotinylated protein in *E. coli* is 22 kDa (14).

Another system that has been used in the literature termed click chemistry, would allow for GPAs with only the benzophenone moiety to be effective. This approach utilizes an alkyne group in place of the relatively bulky Lysine-Biotin-BODIPY group. The benzophenone-GPA could be incubated and photolyzed as we did, but then the tag can be added to the alkyne group under click chemistry conditions (uses copper II salts under reducing conditions such as ascorbic acid to catalyze stereospecific "ligation" of the azide) after the GPA is covalently attached to the proteins of interest (59). This

smaller group may interfere less with binding if in fact that that is the problem with our system.

4.0 Final Conclusions and remarks

The emergence of vancomycin resistant pathogens such as VRSA makes the need for novel antibiotics very real. Three approaches to overcoming GPA resistance have been discussed in this paper.

The first was modified decoration of GPA backbones and has led to the development of Oritavancin and Telavancin, semi-synthetic GPA derivatives that show activity against VRE. Although work in our lab does focus on some GPA modification enzymes, it is not the focus of this thesis.

The second approach is creating modified D-Ala-D-Ala binding pockets. By altering the heptapeptide backbone to replace a carbonyl group thought to be involved in the unfavourable interaction between GPAs and D-Ala-D-Lac with more favourable moieties, groups have developed novel GPA-like compounds that can indeed evade the molecular mechanism of GPA resistance by binding to D-Ala-D-Lac terminating peptidoglycan intermediates. Total synthesis of these complex compounds is not amenable to mass production and so a primary goal of the work presented in this thesis was to expand our knowledge of the molecular events of GPA backbone production. Through deeper understanding of the NRPS backbone assembly and P450-mediated cross-linking we hope to someday use a genetic approach to enable these enzymes to produce compounds with diverse peptide binding pockets that may be suitable backbones for further development of novel antibiotics. Although work on the P450s was halted after *in vitro* activity for OxyB had been reported, the co-expression system for ferredoxins, ferredoxin reductases and P450s may prove to be valuable in future work

with teicoplanin-class GPA backbones once more is known about the vancomycin-class where work is certainly further advanced. It will still be interesting to explore the timing of the StaG-mediated cross-link (the P450 not presenting the vancomycin-class GPA clusters) for development of modified teicoplanin-class backbones.

The final approach to evading GPA resistance explored in this thesis is the inhibition of the resistance mechanism itself. Our hypothesis is that there are GPA-detecting proteins that activate the resistance mechanism and if we can find compounds that bind these proteins but do not activate resistance, we would have promising leads for combination therapies. Our approach was to first identify GPA-binding proteins in GPA producers (that are resistant to GPAs) and then characterize those that are directly involved in GPA resistance. We utilized GPA derivatives bearing the photolabile benzophenone group to covalently cross-link the GPAs to the binding proteins and attempted two methods of detection. The first method was direct detection of fluorescent GPA derivatives. This method showed promise in that it seemed we could detect proteins of similar size to PBPs (that we expected to bind GPAs to some extent). The low intensity signals detected by this method prompted us to explore enriching for the bound proteins using streptavidin resin to pull down proteins that bound biotinylated GPA derivatives. We also opted to amplify the weak signals using anti-biotin Western blots. Initial experiments were promising in that high intensity bands with seemingly dose-dependent signals that could be competed away with high levels of normal vancomycin. Puzzling was the presence of a signal when the samples were never treated with biotinylated GPA derivatives. Despite several rounds of troubleshooting and

verification of these results, we could not eliminate the background signal and have thus opted to explore other approaches to test this hypothesis.

Despite the lack of any results of immediate impact, the work presented in this thesis will lay the ground-work for future experiments with P450s and for future attempts at identifying and characterizing GPA-binding (or recognizing) proteins in the cell membranes of GPA producers and eventually in virulent bacterial strains. Both approaches to evading GPA resistance will almost certainly be the focus of attention in the near future both in our lab and others.

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